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ORIGINAL RESEARCH

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Prognostic impact of molecular subgroups in grade 3 endometrioid endometrial carcinoma: a single cohort study in Northern China

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Abstract

Background: To evaluate the prognostic impact of the molecular classification of grade 3 endometrioid endometrial carcinoma (G3-EEC) and its correlation with clinicopathological factors. **Methods**: 137 patients with G3-EEC were enrolled and molecularly classified using Sanger sequencing in exons 9–14 of the polymerase epsilon (*POLE*) gene and immunohistochemistry (IHC) staining for p53, MLH1 (mutL homolog 1), PMS2 (PMS1 homolog 2), MSH2 (mutS homolog 2) and MSH6 (mutS homolog 6). The Kaplan-Meier method and Cox regression were used for survival analysis, and the chi-square and Fisher's exact tests were used for comparisons between categorical data. **Results**: *POLE* hotspot mutations (*POLE*mut group) were identified in seven tumors (5.1%); 46 (33.6%) tumors showed deficient mismatch repair (dMMR group); 22 (16.1%) showed abnormal p53 staining (p53abn group); and 58 (42.3%) were classified as nonspecific molecular profiles (NSMP group). The remaining four patients (2.9%) were grouped as multi-classifiers. The median follow-up was 45.2 months (range: 6–128 months), with an overall survival (OS) rate of 84.6% (116/137) and a progression-free survival (PFS) rate of 81.7% (112/137). There was no significant difference in the molecular subgroups with OS and PFS ($p = 0.05$ and $p = 0.162$, respectively). The prognosis was most favorable in the *POLE*mut, intermediate in the dMMR and NSMP, worst in the p53abn group, and unclear in the multi-classifier group. Multivariate analysis showed that the International Federation of Gynecology and Obstetrics (FIGO) stage (FIGO 2009 and FIGO 2023) and lymphovascular space invasion were significant independent prognostic factors in OS and PFS ($p < 0.05$). Myometrial invasion, bizarre atypia, and peritumoral lymphocytes showed significant differences among the molecular groups ($p = 0.026$, $p < 0.001$ and $p = 0.001$, respectively). **Conclusions**: Our study demonstrated the prognostic value of the molecular classification of G3-EEC. The biological behavior of the multi-classifier group remains undefined.

Keywords

Prognostic impact; Molecular classification; Grade 3 endometrioid endometrial carcinoma

1. Introduction

Endometrial carcinoma (EC) is the most common gynecologic malignancy after cervical cancer, and third in mortality after cervical and ovarian cancers [1]. Traditional histopathological characteristics, such as tumor type, grade, myometrial invasion and lymphovascular space invasion (LVSI), are crucial in providing a prognostic risk stratification and surgical and adjuvant therapy guidelines for EC [2]. Based on the relation between EC and estrogen, endometrioid EC (EEC) was classified as type I and type II EC, including serous carcinoma, clear cell carcinoma and other non-endometrioid types. The International Society of Gynaecological Pathologists (ISGyP) has recommended a binary grading, which considers the International Federation of Gynaecology and Obstetrics (FIGO) grades 1 and 2 EEC as low-grade, grade 3 endometrioid EC (G3-EEC), and other non-endometrioid endometrial carcinomas as high-grade [3]. However, owing to the heterogeneity, overlapping morphological features and considerable diagnostic interobserver variability in EC [4–6], particularly in high-grade EC, typing and grading do not always lead to accurate prognosis prediction or effective therapy in long-term clinical practice. To provide a more objective method based on molecular levels, The Cancer Genome Atlas (TCGA) study, in 2013, proposed genomic subcategories of EC based on prognostic significance [7]. In this study, 373 EC samples, including 307 EECs, 53 serous carcinomas, and 13 mixed carcinomas, were classified into the *POLE* ultramutated (DNA polymerase epsilon,

*POLE*mut) (7%), microsatellite instability (MSI, hypermutated) (28%), copy number low (CNL)/microsatellite stability (39%) and copy number high (CNH)/serous-like groups (26%). *POLE*mut tumors showed the best outcomes while the CNH group characterized by *Tumor Protein P53* (*TP53*) gene mutation had the worst prognosis. The MSI and CNL groups had an intermediate prognosis. Furthermore, several retrospective studies have confirmed the good prognosis of the *POLE*mut EC [8–10].

However, the TCGA approach has low practicability in routine practice due to its high costs and complex molecular interpretation. The Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) classification was proposed and validated as a surrogate method for clinical practice with prognostic and predictive implications similar to those of the TCGA molecular subtypes [11–13]. This alternative classification was used following sequencing in exons 9–14 of *POLE* exonuclease domain mutations (EDM) to determine the *POLE*mut group and mismatch repair (MMR) proteins (MLH1, MSH2, MSH6 and PMS2). Moreover, p53 immunohistochemical (IHC) staining, instead of molecular testing, was used to determine the MSI and CNH groups, while the remaining tumors were classified as the nonspecific molecular profile (NSMP) group. In addition, molecular subclasses may influence and guide clinical adjuvant treatments, with no adjuvant therapy in stage I–II EC patients with *POLE*mut, and adjuvant treatment for p53-abnormal (p53abn) tumors [2].

Nevertheless, previous studies have yielded inconsistent conclusions regarding the prognostic significance of the molecular classification of EEC. In a real-world cohort, four patients (4/23, 17%) with *POLE*mut EEC developed recurrence: three with initial grade 3 stage I and one with grade 1 stage III disease. One patient with grade 2 stage IV EEC had progressive disease after treatment [14]. Here, we focused on heterogeneous G3-EEC, whose pathogenesis, differential diagnosis, prognosis, and treatment are all still unelucidated. Additionally, G3-EEC can be found in each of the four genomic subgroups. Our study aimed to analyze the prognostic significance of the molecular subtypes of G3-EEC and the differences in clinical outcomes and clinicopathological features in a single-center, large cohort in Northern China.

2. Materials and methods

2.1 Study cohort

Our study comprised a retrospective cohort of patients diagnosed with FIGO G3-EEC, who underwent surgical resection with staging at the Beijing Obstetrics and Gynaecology Hospital, Capital Medical University between January 2010 and December 2020, with a final follow-up date of December 2021. Strict histological diagnostic criteria of G3-EEC were used: (1) solid structural components >50%, containing low-grade EEC components, low-moderate nuclear atypia and/or definitive endometrioid differentiation features, such as squamous differentiation and mucinous differentiation; (2) mixtures of glandular and solid architecture with diffusely high-grade nuclei were excluded. Patients who received neoadjuvant chemotherapy and patients whose paraffin-embedded tissue material was insufficient were also excluded. Among the 146 patients identified, nine cases were excluded because of DNA degradation. Collectively, 137 patients were enrolled in our study cohort.

2.2 *POLE* Sanger sequencing

DNA was extracted from formalin-fixed paraffin-embedded tumor tissue by selecting wax blocks with >60% tumor tissue and <20% necrosis. After quality control and purification, the DNA was amplified using bidirectional primers of exon 9–14 of the *POLE* gene, according to the standard flow. The amplification reaction system was 40 μL of Beijing Jingke Gold mix, which included 35 μL of Gold Brand Mix, 2 μL of upstream primers, 2 μL of downstream primers (20 μmol/L) and 1 μL of DNA. The reaction conditions included pre-denaturation at 98 °C for 2 min and 35 cycles of polymerase chain reaction (PCR). Each PCR cycle comprised denaturation at 98 °C for 10 s, annealing at 60 °C for 10 s, extension at 72 °C for 10 s, and final total extension at 72 °C for 2 min. A single band of amplification was observed when the 2-μL PCR products were subjected to 1.5% agarose gel electrophoresis. Sanger sequencing was performed using an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing results were analyzed using Mutation Surveyor software (Version 5.1.2, Softgenetics, State College, PA, USA). According to previous studies, tumors should be categorized into the *POLE*mut group only when a pathogenic variant (P286R, V411L, S297F, A456P, S459F, F367S, L424I, M295R, P436R, M444K, D368Y and T278M) was found in *POLE* EDM [9, 13, 15].

2.3 IHC staining and molecular subtypes

We performed IHC staining on whole sections of the same blocks to identify MMR (MLH1, PMS2, MSH2 and MSH6) and p53 proteins. The first antibody was stained with rabbit anti-human monoclonal antibodies PMS2 (EP51), MSH2 (RED2), and MSH6 (EP49), and mouse anti-human monoclonal antibodies MLH1 (ES05) and p53 (DO7) (24100909, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd, Beijing, China). Normal endometrium, mesenchymal cells, myometrium and lymphocytes in the sections were used as internal controls. The absence of nuclear expression of any MMR proteins was considered deficient MMR (dMMR). Abnormal p53 staining was defined as strong diffuse staining in >75% of tumor cells, complete absence of staining of tumor cell nuclei or significant cytoplasmic staining. All tumors were divided into molecular groups according to the results of *POLE* mutation detection and IHC staining, and the relevant clinicopathological features of each group were summarized.

2.4 Statistical analysis

Chi-square (χ^2) and Fisher's exact tests were used to evaluate the correlation between molecular subgroups and different clinicopathological features. Kaplan–Meier curves and log-rank statistics were used to determine differences in the overall survival (OS) and progression-free survival (PFS) across

molecular subgroups, and Cox proportional hazard models were used to estimate hazard ratios (HRs) for OS and PFS by univariate and multivariate analyses. The significance level was set as $p < 0.05$. Analyses were performed using IBM SPSS, version 25.0 (IBM SPSS Statistics, Orlando, FL, USA).

3. Results

3.1 Clinical and morphological observations

Our cohort included 137 patients with G3-EEC. The patients' clinical and pathological features are summarized in Tables 1 and 2, respectively. The median age of the patients was 55.1 years (30–73 years). The FIGO 2009 stage distribution was as follows: Ia, 36.4% (50/137); Ib, 28.4% (39/137); II, 11.6% (16/137); IIIa, 4.3% (6/137); IIIb, 2.9% (4/137); IIIc, 13.1% (18/137); IVb, 2.9% (4/137). After adjusting for the FIGO 2023 staging criteria, 11 of the 89 stage I (FIGO 2009) patients without myometrial invasion were further classified as stage Ic (FIGO 2023), 78 patients with any myometrial invasion were categorized as stage IIc, and the staging of stage III and IV patients remained unchanged [16]. Early stages (stages I and II) symptoms were reported by 105 patients, whereas advanced stages (stages III and IV) symptoms were reported by 32 patients. A total of 115 (83.2%) patients received adjuvant therapy, including chemotherapy alone (n = 48), radiotherapy alone (n = 7) or chemotherapy and radiotherapy (n = 60). Two patients with a history of cervical squamous cell carcinoma underwent adjuvant chemoradiotherapy.

The follow-up time was 6–128 months, with a median of 45.2 months. The OS rate of the patients was 84.6% (116/137). Disease progression occurred in 25 patients with pelvic recurrence or metastasis being the most common form of progression (12/25, 48%), followed by vaginal recurrence (5/25, 20%). Vulvar and abdominal wall recurrence each occurred in one patient, respectively. The most frequent site of distant metastases was the lungs (5/25, 20%), followed by the liver (2/25, 8%), brain and bone (1/25, 4%), scalp (1/25, 4%) and the legs (1/25, 4%). One patient presented with multiple metastases throughout the body.

Morphologically, we assessed the myoinvasive patterns and bizarre atypia of nuclear and peritumoral lymphocytes in patients with G3-ECC (Table 2). Four patterns of myoinvasion were observed, excluding the adenoma malignum pattern. The predominant myoinvasive patterns were the infiltrative

TABLE 1. Clinical characteristics of G3-EECs.

Variable		Total N = 137
Median age		55.1 yr (range 30–73 yr)
FIGO Stage (2009)		
	I	89 (65.0%)
	II	16 (11.6%)
	III	28 (20.4%)
	IVb	4 (2.9%)
FIGO Stage (2023)		
	I	11 (8.0%)
	II	84 (61.3%)
	III	28 (20.4%)
	IVb	4 (2.9%)
Adjuvant therapy		
	No	22 (16.1%)
	Chemotherapy alone	48 (35.0%)
	Radiotherapy alone	7 (5.1%)
	Both chemo/radiotherapy	60 (43.8%)
Follow-up time (median)		45.2 mon (range 6–128 mon)
	NED	110 (80.2%)
	AWD	6 (4.4%)
	DOD	21 (15.3%)
Progression		
	No	112 (81.7%)
	Yes	25 (18.3%)

Abbreviations: NED, no evidence of disease; AWD, alive with disease; DOD, died of disease; FIGO, International Federation of Gynaecology and Obstetrics.

TABLE 2. Pathological characteristics of G3-EECs.

Variable	Total N = 137
Myoinvasive pattern	
Non infiltrative	11 (8.0%)
MELF	14 (10.2%)
Infiltrating glands	72 (52.6%)
Broad front	27 (19.7%)
Adenomyosis-like	13 (9.5%)
Bizarre atypia	
No	124 (90.5%)
Yes	13 (9.5%)
Peritumoral lymphocytes	
No	36 (26.3%)
Yes	101 (73.7%)
Myometrial invasion	
No	11 (8.0%)
$<1/2$	51 (37.2%)
$\geq 1/2$	75 (54.8%)
Endocervical stromal invasion	
No	101 (73.7%)
Yes	36 (26.3%)
LVSI	
No or focal	77 (56.2%)
Substantial	60 (43.8%)
Lymph node metastases	
No	114 (83.2%)
Yes	23 (16.8%)
Adnexal metastases	
No	127 (92.7%)
Yes	10 (7.3%)
Positive peritoneal cytology*	
No	98 (80.3%)
Yes	24 (19.7%)
ER	
Negative	27 (19.7%)
Positive	110 (80.3%)
PR	
Negative	35 (25.5%)
Positive	102 (74.5%)

**15 cases did not receive washing for peritoneal cytology. MELF, microcystic elongated and fragmented glands; LVSI, lymphovascular space invasion; ER, estrogen receptor; PR, progesterone receptor.*

glands (52.6%), broad front (19.7%), microcystic elongated and fragmented glands (MELF) (10.2%) and adenomyosis-like patterns (9.5%). Moreover, only 11 cases (8.0%) showed non-infiltrative myoinvasion. Collectively, 13 tumors (9.5%) with bizarre atypia and 101 (73.7%) with peritumoral lymphocytes were found (Fig. 1). Substantial LVSI (≥5 vessels) was found in 60 cases (43.8%). Tumors were positive and negative for the estrogen receptor (ER) in 110 (80.3%) and 27 cases (19.7%), respectively, while 102 (74.5%) and 35 cases (25.5%) were positive and negative for the progesterone receptor (PR), respectively.

3.2 Molecular subgroups

POLE EDM hotspot mutations, including P286R and F367S substitutions (five and two cases, respectively), and one case each of S297F, V411L and T278M substitutions, were found in 10 cases (10/137, 7.2%). However, 13 variants of unknown clinical significance were found, namely N336S and T308I in exon 10; D368N, P370S, G388C, and S393G in exon 11; P436T and M444I in exon 13; and T454I, V474, I484N, M487V, and M489S in exon 14.

Among the 50 dMMR cases (50/137, 36.5%), 35 tumors showed loss of MLH1 and PMS2. Four cases showed losses of both MSH2 and MSH6; three cases showed loss of MSH2 alone; six cases showed loss of MSH6 alone; and the final two cases showed loss of PMS2 alone. We did not perform further testing for germline mutations in these cases, and only one patient had Lynch syndrome carrying a germline mutation in the *MSH2* gene.

Twenty-five tumors (25/137, 18.2%) showed p53abn expression, of which 21 showed strong diffuse p53 staining as missense mutations and four tumors showed no staining for p53 as nonsense mutations (Fig. 1). No case showed abnormal cytoplasmic expression.

Among the four multi-classifier cases, one with *POLE*mut (F367S) showed both dMMR (MLH1 and PMS2 loss) and p53abn staining (Fig. 2); one case exhibited *POLE*mut (T278M) and dMMR (MLH1 and PMS2 loss); one *POLE*mut (F367S) tumor displayed p53abn staining; and one tumor showed dMMR (MSH2 loss) and p53 missense expression but no *POLE* mutation. Fifty-eight cases showed no abnormalities and were classified as NSMP. Finally, seven cases (7/137, 5.1%) were classified as the *POLE*mut group, 46 cases (46/137, 33.6%) as the dMMR group, 22 cases (22/137, 16.1%) as the p53abn group (22/137, 16.1%), 58 cases (58/137, 42.3%) as the NSMP group and four cases (4/137, 2.9%) as the multi-classifier group.

3.3 Relationships between molecular subgroups and clinicopathological parameters

The univariate associations of molecular groups with clinicopathological characteristics were calculated and summarized in Table 3. Significant differences in myometrial invasion, bizarre atypia, and peritumoral lymphocytes were observed among different molecular groups ($p = 0.026$, $p = 0.001$ and $p < 0.001$, respectively). Peritumoral lymphocyte infiltration and bizarre atypia were common in the *POLE*mut group (Fig. 1). Peritumoral lymphocyte infiltration was more likely to be found in the dMMR group, and bizarre atypia was common in the p53abn group. No significant differences were observed between the myoinvasion patterns and molecular groups. Among myoinvasion patterns, non-infiltrative myoinvasion did not appear in the *POLE*mut group. The MELF pattern was only observed in 14 cases, possibly due to fewer glandular structures in G3-EEC cases than in low-grade EEC. Other clinicopathological features, such as age, endocervical stromal invasion, LVSI, lymph node metastases, adnexal metastases, FIGO stage, positive peritoneal cytology and ER and PR staining, showed no significant differences by molecular subtypes ($p > 0.05$).

3.4 Survival analysis results

For OS, no significant differences were observed across the molecular subgroups ($p = 0.050$), and clinical outcomes showed a very similar distribution to the original TCGA categories (Fig. 3). The prognosis was most favorable in the *POLE*mut group, worst in the p53abn group, and intermediate in the dMMR and NSMP groups, while the prognosis trend in the multi-classifier group was unclear. However, the association between the molecular groups and PFS was not statistically significant ($p = 0.162$). In addition, univariate survival analysis showed that all clinicopathological variables of prognostic importance for EC were significantly associated with OS time in this cohort, such as FIGO stage (FIGO 2009 and FIGO 2023), lymph node metastasis, adnexal metastasis, myometrial invasion, endocervical stromal invasion, LVSI and positive peritoneal cytology ($p < 0.05$) (Table 4). Patients with ER/PR-negative tumors had a shorter OS. Furthermore, FIGO stage, lymph node metastasis, adnexal metastasis, LVSI and positive peritoneal cytology were significantly different in PFS ($p < 0.05$). Multivariate analysis using the Cox proportional hazards model showed that FIGO stage (2009/2023) ($p < 0.001$) and LVSI ($p = 0.042$, HR = 3.172, 95% Confidence Interval (95% CI): 1.041–9.668) were significant independent prognostic factors for clinical outcomes in OS. Additionally, the FIGO stage (2009/2023) ($p < 0.05$) and LVSI ($p = 0.036$, HR = 2.915, 95% CI: 1.075–7.905) were significant independent prognostic factors in PFS. However, molecular subtypes could not be validated as independent prognostic parameters in either category.

4. Discussion

This study used simultaneous Sanger sequencing of *POLE* EDM and IHC staining for p53, MLH1, MSH2, MSH6 and PMS2 molecular classification in 137 G3-EECs. Moreover, we assessed the prognostic significance of molecular subtypes and associated clinicopathological features. Our findings revealed that molecular subgroups had distinct favorable prognostic predictive values but were not independent prognostic factors for G3-EEC. As a high-grade adenocarcinoma, G3-EEC is the least represented of all EECs, with this study including the largest number of G3-EEC patients in any single-center study to date, to our knowledge.

Only 10 cases of *POLE* EDM were detected in G3-EEC

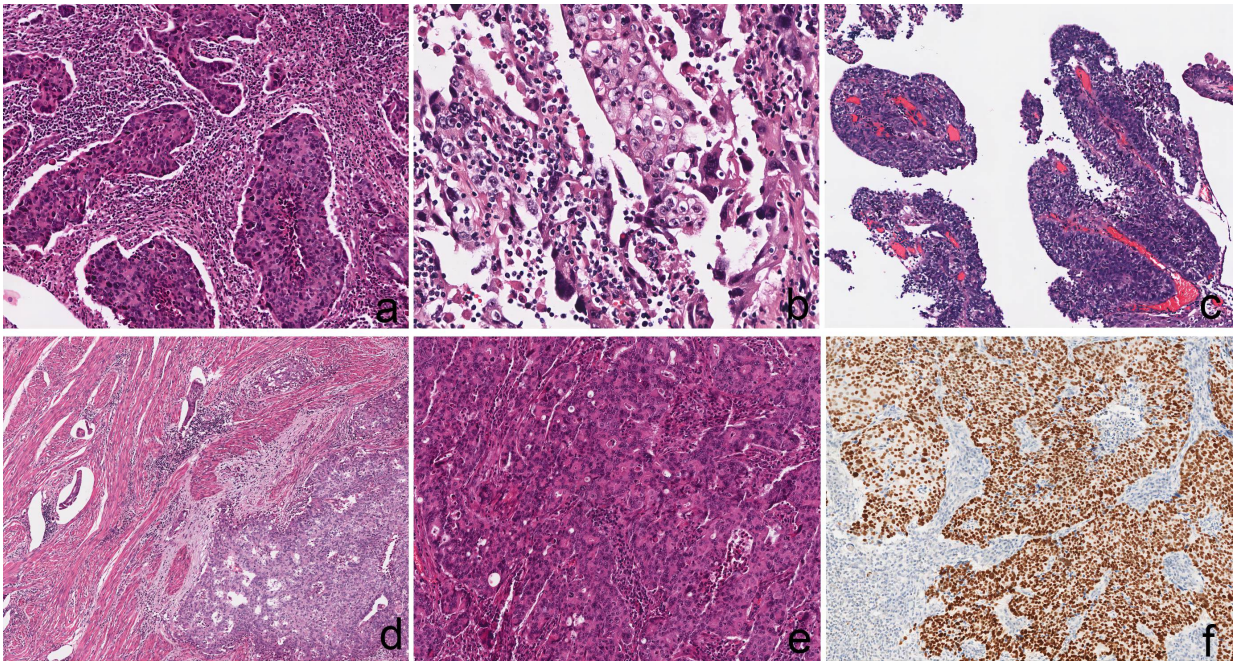

FIGURE 1. G3-EECs. (a) Peritumoral lymphocyte infiltration (×200), (b) bizarre atypia (×400), (c) tumor cell growth around vessels (×100), (d) deep myometrial invasion and LVSI (×100), (e,f) the same case displayed with solid architecture and abnormal staining of p53 (×200), (f) p53 staining was diffused positive. Abbreviations: G3-EEC, grade 3 endometrioid endometrial carcinoma; LVSI, lymphovascular space invasion.

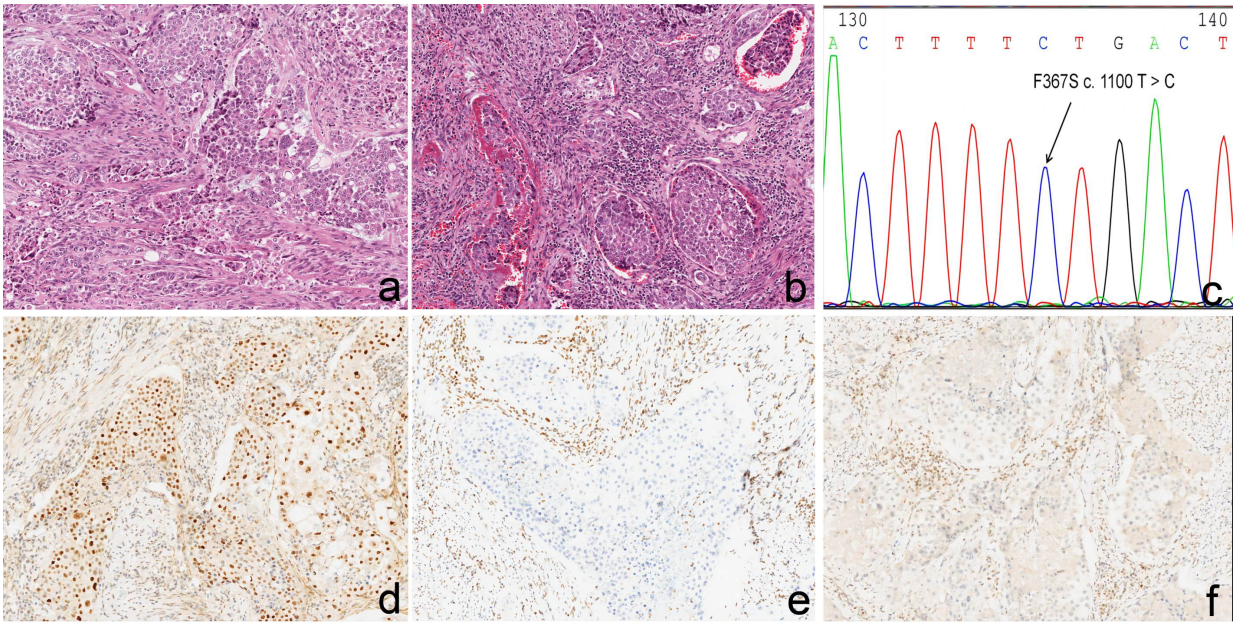

FIGURE 2. A multi-classifier case of G3-EEC. (a) Poorly differentiated tumor cells (×200), (b) LVSI (×200), (c) *POLE*mut (F367S), (d) diffuse positive staining for p53, (e) loss of staining for MLH1, (f) loss of staining for PMS2 (×200). Abbreviation: G3-EEC, grade 3 endometrioid endometrial carcinoma; LVSI, lymphovascular space invasion.

patients (10/137, 7.2%). Three cases were classified as a multi-class group because they simultaneously carried other molecular alterations, leaving only seven cases (5.1%) in the *POLE*mut group. This incidence is in slight contrast with previous reports in the literature. A Canadian study reported that the *POLE* mutation rate was 15% in G3-EEC cases (8/53) [8]. A large and well-characterized multicenter study of G3-EEC from six centers in North America and Europe revealed a *POLE* mutation rate of 12.9% (49/381) [17]. More recently, in a larger single-center study that included 95 G3-EECs, 10 cases (11%) of *POLE* EDM were identified [18]. However, one study from China including 46 G3-EEC cases revealed a *POLE* mutation rate of 6.9% [19]. Another similar study found a *POLE* mutation rate of 7.5%, with only 62 G3-EEC cases [20]. The rate of *POLE* mutations in EEC in a Japanese study [21] was 4.6%, similar to the results of this study. Presumably, the differences in the detection rate of *POLE* mutations and mutation hotspots may be correlated with genetic background

TABLE 3. Clinicopathological characteristics of the molecular groups.

Variable	Total N = 137					
	*POLE*mut N = 7 (5.1%)	dMMR N = 46 (33.6%)	p53abn N = 22 (16.1%)	NSMP N = 58 (42.3%)	Multi Class N = 4 (2.9%)	*p*
Age						
<55 yr	2 (28.6%)	20 (43.5%)	8 (36.4%)	24 (41.4%)	1 (25.0%)	0.913
≥55 yr	5 (71.4%)	26 (56.5%)	14 (63.6%)	34 (58.6%)	3 (75.0%)	
Myometrial invasion						
No	0 (0.0%)	2 (4.3%)	2 (9.1%)	6 (10.5%)	1 (25.0%)	
<1/2	6 (85.7%)	18 (38.3%)	12 (54.5%)	15 (26.3%)	0 (0.0%)	0.026
≥1/2	1 (14.3%)	27 (57.4%)	8 (36.4%)	36 (63.2%)	3 (75.0%)	
Endocervical stromal invasion						
No	7 (100.0%)	31 (67.4%)	16 (72.7%)	44 (75.9%)	3 (75.0%)	0.474
Yes	0 (0.0%)	15 (41.7%)	6 (16.7%)	14 (24.1%)	1 (25.0%)	
LVSI						
No or focal	4 (57.1%)	22 (47.8%)	13 (59.1%)	35 (60.3%)	3 (75.0%)	0.691
Substantial	3 (42.9%)	24 (52.2%)	9 (40.9%)	23 (39.7%)	1 (25.0%)	
Lymph node metastases						
No	7 (100.0%)	36 (78.3%)	18 (81.8%)	49 (84.5%)	4 (100.0%)	0.698
Yes	0 (0.0%)	10 (21.7%)	4 (18.2%)	9 (15.5%)	0 (0.0%)	
Adnexal metastases						
No	7 (100.0%)	43 (93.4%)	18 (81.8%)	55 (94.8%)	4 (100.0%)	0.374
Yes	0 (0.0%)	3 (6.6%)	4 (18.2%)	3 (5.2%)	0 (0.0%)	
FIGO stage (2009)						
I	7 (100.0%)	27 (58.7%)	13 (59.1%)	39 (67.2%)	3 (75.0%)	
II	0 (0.0%)	6 (13.0%)	1 (4.5%)	8 (13.8%)	1 (25.0%)	0.348
III	0 (0.0%)	12 (26.1%)	8 (36.4%)	8 (13.8%)	0 (0.0%)	
IV	0 (0.0%)	1 (2.2%)	0 (0.0%)	3 (5.2%)	0 (0.0%)	
FIGO stage (2023)						
I	0 (0.0%)	2 (4.3%)	2 (9.1%)	6 (10.3%)	1 (25.0%)	
II	7 (100.0%)	31 (67.4%)	12 (54.5%)	41 (70.7%)	3 (75.0%)	0.306
III	0 (0.0%)	12 (26.2%)	8 (36.4%)	8 (13.8%)	0 (0.0%)	
IV	0 (0.0%)	1 (2.1%)	0 (0.0%)	3 (5.2%)	0 (0.0%)	
Positive peritoneal cytology*						
No	6 (85.7%)	32 (78.0%)	12 (66.7%)	44 (84.6%)	4 (100.0%)	0.485
Yes	1 (14.3%)	9 (22.0%)	6 (33.3%)	8 (15.4%)	0 (0.0%)	
Myoinvasive pattern						
Non infiltrative	0 (0.0%)	2 (4.3%)	2 (9.1%)	6 (10.3%)	1 (25.0%)	
MELF	2 (28.6%)	3 (6.5%)	3 (13.6%)	6 (10.3%)	0 (0.0%)	
Infiltrating glands	2 (28.6%)	33 (71.8%)	8 (36.4%)	25 (43.1%)	3 (75.0%)	0.218
Broad front	2 (28.6%)	6 (13.1%)	7 (31.8%)	13 (22.4%)	0 (0.0%)	
Adenomyosis-like	1 (14.3%)	2 (4.3%)	2 (9.1%)	8 (13.9%)	0 (0.0%)	
Bizarre atypia						
No	5 (71.4%)	45 (97.8%)	15 (68.2%)	57 (98.3%)	2 (50.0%)	<0.001
Yes	2 (28.6%)	1 (2.2%)	7 (31.8%)	1 (1.7%)	2 (50.0%)	

TABLE 3. Continued.

Variable	Total N = 137					
	*POLE*mut N = 7 (5.1%)	dMMR N = 46 (33.6%)	p53abn N = 22 (16.1%)	NSMP N = 58 (42.3%)	Multi Class N = 4 (2.9%)	*p*
Peritumoral lymphocytes						
No	0 (0.0%)	4 (8.7%)	9 (40.9%)	22 (37.9%)	1 (25.0%)	0.001
Yes	7 (100.0%)	42 (91.3%)	13 (59.1%)	36 (62.1%)	3 (75.0%)	
ER						
Negative	2 (28.6%)	9 (19.6%)	5 (22.7%)	10 (17.2%)	1 (25.0%)	0.846
Positive	5 (71.4%)	37 (80.4%)	17 (77.3%)	48 (82.8%)	3 (75.0%)	
PR						
Negative	3 (42.9%)	11 (23.9%)	5 (22.7%)	15 (25.9%)	1 (25.0%)	0.854
Positive	4 (57.1%)	35 (76.1%)	17 (77.3%)	43 (74.1%)	3 (75.0%)	

**15 cases did not receive washing for peritoneal cytology. NSMP, nonspecific molecular profiles; LVSI, lymphovascular space invasion; FIGO, International Federation of Gynaecology and Obstetrics; MELF, microcystic elongated and fragmented glands; ER, estrogen receptor; PR, progesterone receptor.*

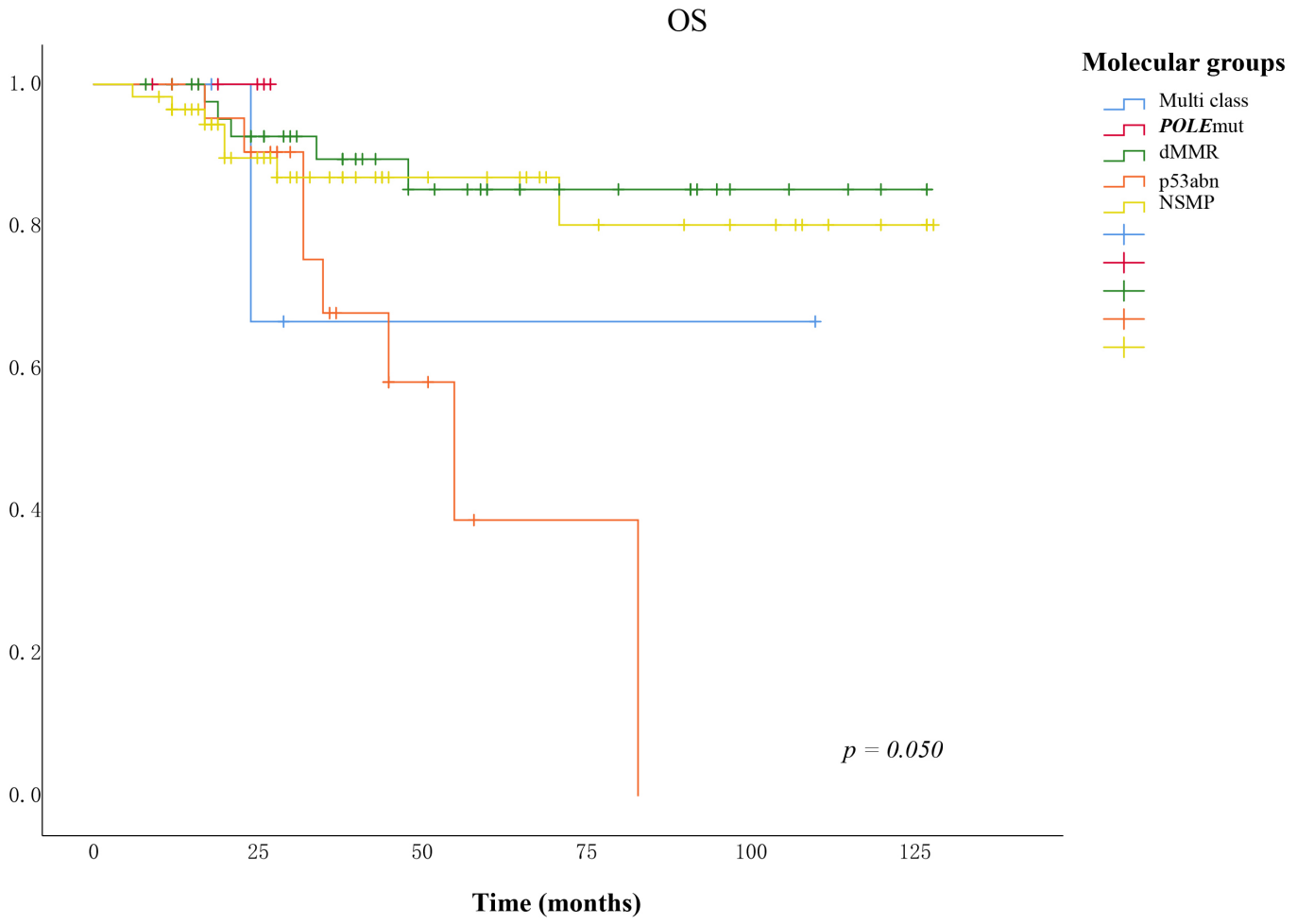

FIGURE 3. Kaplan-Meier survival curves by molecular groups among G3-EEC patients for overall survival. Abbreviation: G3-EEC, grade 3 endometrioid endometrial carcinoma; *POLE*mut, *POLE* mutation; dMMR, deficient mismatch repair; p53abn, p53 abnormal; NSMP, nonspecific molecular profile; OS, overall survival.

TABLE 4. Univariate and multivariate analysis of survival in patients with G3-EECs.

Variable	OS				PFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age (yr)	0.998 (0.949–1.049)	0.944			1.010 (0.965–1.057)	0.668		
Myometrial invasion								
No	0.000 (0.000)				0.000 (0.000)			
<1/2	0.290 (0.098–0.864)	0.014			0.236 (0.081–0.689)	0.003		
≥1/2	1				1			
Endocervical stromal invasion								
No	1	0.010			1	0.280		
Yes	1.792 (1.150–2.792)				1.582 (0.680–3.679)			
Lymph node metastases								
No	1	<0.001			1	<0.001		
Yes	7.038 (2.816–17.589)				4.568 (2.024–10.310)			
Adnexal metastases								
No	1	<0.001			1	0.001		
Yes	8.239 (3.128–21.703)				4.334 (1.617–11.619)			
LVSI								
No or focal	1	0.008	1	0.042	1	0.001	1	0.036
Substantial	2.126 (1.213–3.725)		3.172 (1.041–9.668)		3.893 (1.624–9.334)		2.915 (1.075–7.905)	
Positive peritoneal cytology								
No	1	0.020	1	0.166	1	0.036	1	0.598
Yes	2.966 (1.190–7.390)		2.714 (0.661–11.142)		2.462 (1.032–5.872)		0.737 (0.237–2.291)	
FIGO Stage (2009)								
I	1		1		1		1	
II	0.000 (0.000)	<0.001	0.000 (0.000)	<0.001	0.000 (0.000)	<0.001	0.000 (0.000)	0.018
III	8.887 (3.239–24.384)		29.200 (1.740–489.961)		3.572 (1.517–8.412)		4.806 (1.622–14.242)	
IV	9.275 (1.918–44.865)		16.133 (0.787–330.826)		7.118 (1.999–25.339)		14.570 (1.651–128.586)	
FIGO Stage (2023)								
I	0.000 (0.000)		0.000 (0.000)		0.000 (0.000)		0.000 (0.000)	
II	0.106 (0.022–0.513)	<0.001	0.030 (0.002–0.470)	<0.001	0.134 (0.138–0.478)	<0.001	0.108 (0.017–0.685)	0.006
III	0.976 (0.205–4.648)		0.793 (0.069–9.145)		0.505 (0.138–1.854)		0.524 (0.081–3.387)	
IV	1		1		1		1	

TABLE 4. Continued.

Variable	OS				PFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Molecular groups								
*POLE*mut	0.000 (0.000)		0.000 (0.000)		0.000 (0.000)			
dMMR	0.346 (0.040–2.976)	0.050	0.145 (0.015–1.441)	0.060	0.697 (0.088–5.505)	0.162		
p53abn	1.454 (0.179–11.781)		1.114 (0.109–11.337)		1.356 (0.169–10.857)			
NSMP	0.462 (0.057–3.769)		0.208 (0.020–2.167)		0.442 (0.054–3.593)			
Multi Class	1		1		1			
Adjuvant Treatment								
No	1	0.184			1	0.089		
Yes	3.911 (0.523–29.217)				4.805 (0.650–35.526)			
ER								
Negative	1	0.002	1	0.262	1	0.076	1	0.573
Positive	0.261 (0.111–0.615)		0.471 (0.126–1.757)		0.476 (0.205–1.103)		0.719 (0.228–2.266)	
PR								
Negative	1	0.005	1	0.908	1	0.069	1	0.885
Positive	0.295 (0.125–0.695)		1.093 (0.240–4.985)		0.484 (0.217–1.077)		1.099 (0.305–3.956)	

Abbreviations: OS, overall survival; PFS, progression-free survival; LVSI, lymphovascular space invasion; HR, hazard ratios; CI, confidence interval; FIGO, International Federation of Gynaecology and Obstetrics; ER, estrogen receptor; PR, progesterone receptor.

or differing definitions of a pathogenic *POLE* EDM in various studies. Therefore, careful selection of pathogenic mutations is necessary to define this group. Five *POLE* hotspot mutations were identified in the original TCGA categories, including P286R, V411L, S297F, A456P and S459F. Subsequent studies have identified more hotspot mutations. The definition of pathogenic *POLE* EDM and the sequencing exon were different, based on either the complete exonuclease domain (exons 9–14) or targeted sequencing of exons 9, 13 and 14. Cosgrove *et al*. found that no mutations were found in exons 10 and 11 [22], suggesting that the sequencing of exons 9, 12, 13 and 14 was sufficient for diagnosing pathogenic *POLE* mutations. However, the *POLE* genomic alteration score algorithm [15], which is based on the mutation type proportion, tumor mutational burden and variant recurrence in EC, and a *POLE* score of ≥4 has been proposed to define the pathogenicity of *POLE* mutations in EC. Thus, the *POLE* hotspot mutations harbored seven additional unique mutations including F367S, L424I, M295R, P436R, M444K and D368Y, with F367S and D368Y precisely located at exon 11. In a recent study, three additional new hotspot EDMs, namely R375Q, P452L and T278K, were identified in patients with invasive EC subtypes [23, 24]. Thus, we recommend using the complete exonuclease domain (exons 9–14) rather than selective exons for *POLE* mutation detection to avoid missing mutation cases.

The survival curves between the molecular group and OS in our analysis showed a trend towards the best and worst prognosis in the *POLE*mut and p53abn groups, respectively. Among the seven *POLE*mut patients, six were in FIGO stage Ia and one in FIGO stage Ib, all of whom survived without recurrence or metastasis; however, the follow-up period was rather short. Among the 58 patients in the p53abn group, seven had disease progression and died. This is consistent with most findings regarding the molecular typing of EC [7, 8, 11–13, 17, 19, 25]. However, results so far have been inconclusive. Li *et al*. [20] discovered that patients with *POLE* mutations who underwent chemoradiotherapy had poor OS ($p < 0.0001$). Four patients developed recurrences, and one had progressive disease after treatment among 23 *POLE* EDM cases in a real-world cohort [14]. Further research is needed before the presence of *POLE* EDM can be included in decisions about adjuvant therapy.

Based on our findings, the exact classification and prognostic significance of the multi-classifier group were unclear. Some studies supported the classification of *POLE*mut–p53abn EC as *POLE*mut, and dMMR-p53abn EC as dMMR based on prognostic significance [26], suggesting that *TP53* variants occurring in the context of a *POLE*mut or

dMMR EC are likely passenger events rather than pathogenic mutations. Four multi-classifier cases were identified in our study, three combined with abnormal expression of p53. One patient with a stage Ib *POLE*mut (F367S) showing both dMMR (MLH1 and PMS2 loss) and p53abn staining had a recurrence in the pelvis and died 24 months after surgery, while the remaining three patients showed no evidence of disease. In this study, when survival analysis was attempted without the inclusion of the multi-group, a significant difference was observed between each molecular subgroup and OS (p = 0.022) (Fig. 4a); however, no significant difference was observed between each molecular subgroup and PFS (p = 0.091) (Fig. 4b). Survival analysis performed after the multi-classifier group was meticulously reclassified into the *POLE*mut and dMMR groups according to literature [26], also showed no significant difference between the molecular groups and PFS (p = 0.139) (Fig. 5, Ref. [26]). Despite the significant difference between each molecular subgroup and OS (p = 0.030) (Fig. 5a), the "*POLE*mut group" did not have the best prognosis due to one death case in this group. Such a result may be the reason for Joehlin-Price *et al.*'s [18] exclusion of multi-class group cases in their survival analysis. The use of multi-class molecular subgroups is controversial; thus, future studies must focus on the genetic alterations and prognostic significance of these cases.

Next-generation sequencing (NGS) is the first choice for the molecular classification of EC if more genetic information needs to be obtained. However, NGS is expensive and time-consuming. A smaller 11-gene NGS panel that combined mutation and MSI analyses was designed to provide an efficient and accurate molecular classifier for EC [27], that showed excellent accessibility compared to IHC approaches. Considering the need for a better cost-benefit ratio, numerous studies have replaced NGS testing with Sanger sequencing of *POLE* mutations and IHC assays of MMR and p53 proteins. Most studies on molecular test algorithmic steps recommend sequential inertia for each test. However, whether the first step should comprise the ProMisE algorithm with MMR IHC [13] or the algorithm recommended by NCCN guidelines for *POLE* mutation detection [28] remains unclear. Conducting either of the tests alone is insufficient because the multi-classifier cases represent approximately 5% of all cancers. Therefore, we recommend that all tests be performed simultaneously, rather than using the algorithmic approach of the molecular classification system. Since IHC for MMR and p53 proteins is routinely performed in every newly diagnosed EC case in China, the main limiting factor for routine molecular classification currently is *POLE* mutation testing, as some pathological laboratories cannot perform molecular testing routinely. Instead of Sanger sequencing-based approaches, Devereaux *et al.* [29] developed a novel, simpler, inexpensive and highly sensitive SNaPshot assay that interrogates 15 nucleotide sites within exons 9, 11, 13 and 14 encoding the *POLE* exonuclease domain. However, the IHC of p53abn was an imperfect substitute for *TP53* mutation detection. In addition, a small number of high-copy-number tumors did not show *TP53* mutations. A combined analysis of conventional pathology and molecular findings is ideal to minimize these limitations [2].

A meta-analysis of studies providing a histopathological characterization of molecular groups of ECs [30] revealed that in the dMMR group, dMMR-ECs were endometrioid in most cases (85.8%), and showed G3, LVSI and deep myometrial invasion in almost half of the cases. The prevalence of aggressive parameters was lower in the *POLE*mut group than in the dMMR group and was associated with almost no lymph node metastasis. Another meta-analysis concluded that *POLE*mut tumors were more likely to be high-grade, early-stage, limited to the endometrium and have a reduced possibility of lymph node involvement, resulting in improved survival [31]. The NSMP group showed the most favorable clinicopathological profile among the four groups, most of which were low-grade EEC and showed a low prevalence of parameters of aggressiveness. This group accounts for the largest number of patients with unclear molecular features. Subsequent studies have refined the molecular/prognostic classification, demonstrating that high expression of L1 cell adhesion molecule (L1CAM) and β-catenin–encoding gene (CTNNB1) exon 3 mutations have an independent prognostic value [32, 33]. The p53abn

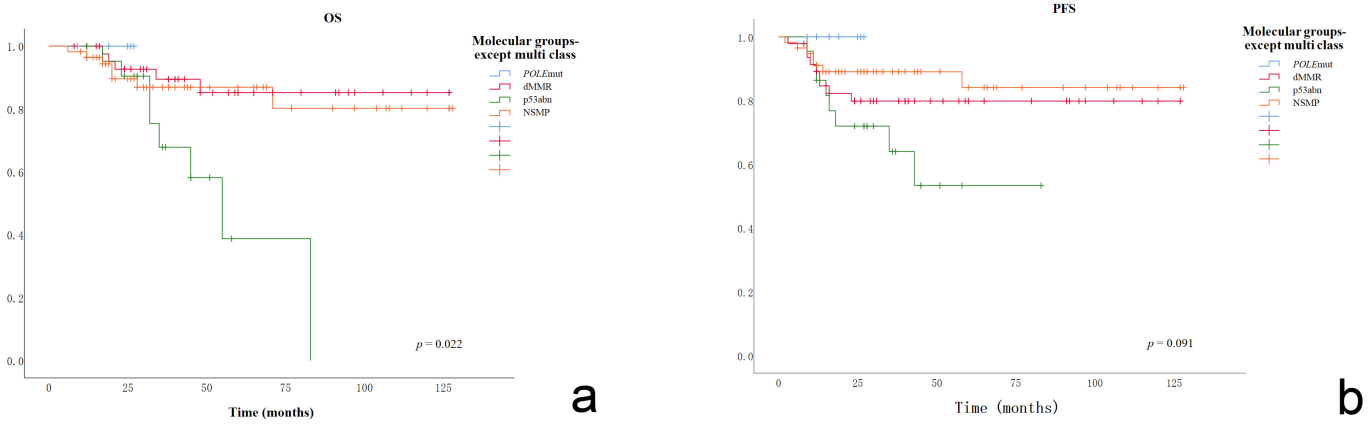

FIGURE 4. Kaplan-Meier survival curves by molecular groups except multi-class among G3-EEC patients for OS (a) and PFS (b), with a *p*-value of 0.022 and 0.091, respectively. Abbreviations: G3-EEC, grade 3 endometrioid endometrial carcinoma; OS, overall survival; PFS, progression-free survival; *POLE*mut, *POLE* mutation; dMMR, deficient mismatch repair; p53abn, p53 abnormal; NSMP, nonspecific molecular profile.

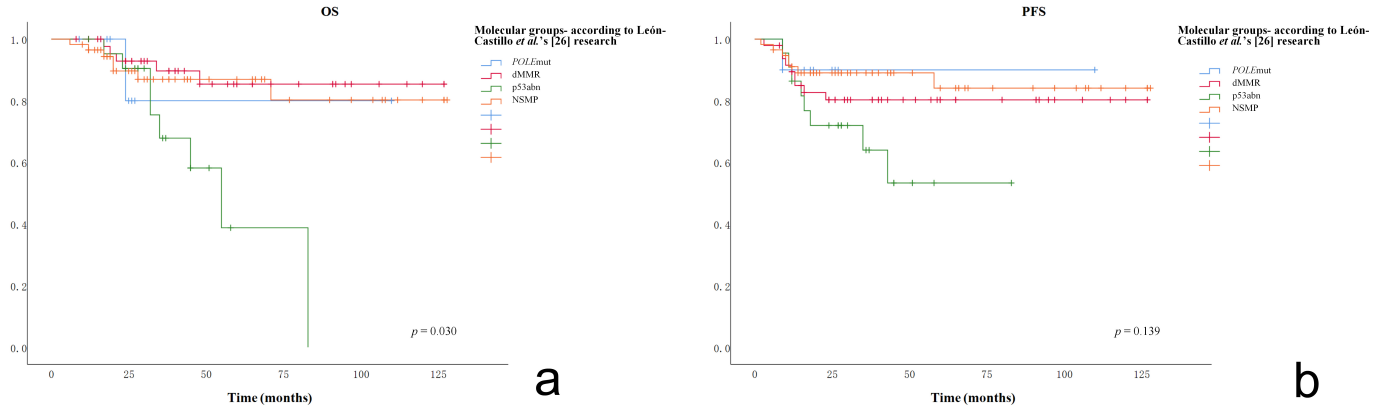

FIGURE 5. Kaplan-Meier survival curves by molecular groups according to León-Castillo *et al.*'s [26] research among G3-EEC patients for OS (a) and PFS (b), with a *p*-value of 0.030 and 0.139, respectively. Abbreviations: G3-EEC, grade 3 endometrioid endometrial carcinoma; OS, overall survival; PFS, progression-free survival; *POLE*mut, *POLE* mutation; dMMR, deficient mismatch repair; p53abn, p53 abnormal; NSMP, nonspecific molecular profile.

group had the worst prognosis, with the highest prevalence of all unfavorable histopathological features and the lowest endometrioid histotype. The present study was limited to all G3-EEC, and only one *POLE*mut tumor showed deep myoinvasion, with no significant difference observed in other clinicopathological characteristics among different molecular groups.

In addition to the conventional prognostic parameters for stratifying risk, we evaluated other features, such as myoinvasive patterns and morphological features in relation to molecular subtypes. Ruz-Caracuel *et al*. [34] found that the predominant broad front myoinvasive pattern was significantly associated with tumor relapse (p = 0.003). The presence of a pattern of infiltrative glands (p = 0.001) and microsatellite instability (p = 0.004) was associated with lower disease-free survival. He *et al*. [19] demonstrated that in *POLE*mut tumors, the MELF invasive pattern was associated with a 15.1-fold increased risk of tumor recurrence or progression. In this study, myoinvasion patterns were not significantly different across molecular subgroups. Peritumoral lymphocytes and bizarre atypia differed significantly across subgroups; however, these features were present to some degree in all groups.

The significance of molecular classification for EC lies not only in predicting prognosis but also in choosing postoperative treatment and chemotherapy regimen selection. In patients with stages I and II EC, adjuvant therapy should be avoided based on the low risk of pathogenic *POLE* mutation [2]. When considering adjuvant chemotherapy in high-grade/high-risk diseases, clinical management may be particularly affected by molecular classification. All stage I–IVA p53abn EC cases in the PORTEC-3 trial were considered high-risk cancers, and adding chemotherapy to the treatment plan was beneficial [35].

Nonetheless, increasing evidence has shown the importance of traditional pathological features, such as histopathologic type, grade, myometrial invasion and LVSI, in predicting prognosis [3]. The European Society of Gynaecological Oncology (ESGO), European Society for Radiotherapy and Oncology (ESTRO) and European Society of Pathology (ESP) guidelines for the management of EC recommend the assessment of molecular signatures as an additional prognostic factor to be integrated with classic pathological factors (such as histotype, myometrial invasion or LVSI) [2]. Histological typing of EC showed an important independent prognostic value in the TCGA subgroup, while non-endometrioid carcinoma had a poor clinical outcome in each TCGA subgroup [36]. A recent systematic review and meta-analysis revealed that deep myometrial invasion was not an independent prognostic factor for OS in EC patients, but was associated with a 1.5–2-fold increased risk of recurrence independently of the TCGA groups [37]. However, LVSI had a prognostic value independent of TCGA characteristics, age, and adjuvant therapy, increasing the risk of death, either due to EC or recurrent or progressive disease by 1.5–2 folds [38]. Our study confirmed that substantial LVSI was an independent prognostic factor for shorter OS and PFS in addition to the advanced FIGO stage. While molecular classification adds another layer of information to traditional morphological features, how these molecular groups can be integrated with other prognostic histological factors in treating EC remains under investigation.

This study had some limitations. First, because of the nature of retrospective cohort studies, confounding variables, such as selection and reporting bias, may negatively affect the accuracy of the results. Second, this study had a relatively small sample size, especially in the *POLE*mut group; however, to date, it is the single-center study with the largest sample size.

5. Conclusions

Our data supports the distinct prognostic differences between the four molecular subgroups in G3-EEC. The prognosis was favorable in the *POLE*mut, intermediate in the dMMR and NSMP and worst in the p53abn groups. However, the biological behavior of the multiple-classifier group remains unclear. A correlation exists between molecular classification and tumor morphology; however, the clinicopathological features that differentiate molecular groups remain unclear. Therefore, we recommend simultaneous molecular surrogate tests in all newly diagnosed ECs in routine clinical practice to screen as many molecular subgroups as possible and observe the genuine

biological behavior of the group through the accumulated data.

AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article.

AUTHOR CONTRIBUTIONS

WXZ and HGL—designed the research study; wrote the manuscript. WXZ—performed the research. JZ, YZ and YLJ—analyzed the data. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Committee of Beijing Obstetrics and Gynecology Hospital (IEC-C-29-V03-FJ1). Informed consent in all cases was obtained from the patients or their relatives at the time of outpatient visits or by mail while the study was in progress.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

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