Clinicopathological characteristics of multiple-classifier endometrial cancers: a cohort study and systematic review

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ABSTRACT

Background Endometrial cancers with more than one molecular feature—*POLE* mutations (POLEmut), mismatch repair protein deficiency (MMRd), p53 abnormality (p53abn)—are called 'multiple classifiers'.

Objective To describe our cohort of multiple classifiers and to report the results of a review on their incidence and the techniques used to identify them.

Methods Multiple classifiers identified at the European Institute of Oncology, Milan, between April 2019 and Decmber 2022, were included. Clinicopathological, molecular characteristics, and oncologic outcomes were summarized and compared between single and multiple classifiers sharing common features. Studies on molecular classification of endometrial cancer were searched in the PubMed Database to collect data on the incidence of multiple classifiers and the techniques used for classification.

Results Among 422 patients, 48 (11.4%) were multiple classifiers: 15 (3.6%) POLEmut-p53abn, 2 (0.5%) POLEmut-MMRd, 28 (6.6%) MMRd-p53abn, and 3 (0.7%) POLEmut-MMRd-p53abn. MMRd-p53abn and MMRd differed in histotype (non-endometrioid: 14.8% vs 2.0%, p=0.006), grade (high-grade: 55.6% vs 22.2%, p=0.001), and MMR proteins expression, whereas they differed from p53abn in histotype (non-endometrioid: 14.8% vs 50.0%, p=0.006). POLEmut-p53abn and POLEmut differed only in grade (high-grade: 66.7% vs 22.7%, p=0.008), while they differed from p53abn in age (56.1 vs 66.7 years, p=0.003), stage (advanced: 6.7% vs 53.4%, p=0.001), and histotype (non-endometrioid: 6.7% vs 50.0%, p=0.002). Two (7.1%) patients with MMRd-p53abn, 4 (4.0%) with MMRd, and 25 (34.3%) with p53abn had a recurrence. No recurrences were observed in POLEmut-p53abn and POLEmut. *TP53* sequencing allowed the detection of additional 7 (18.9%) multiple classifiers with normal p53 immunostaining. The incidence of multiple classifiers ranged from 1.8% to 9.8% in 10 published studies including >100 patients. When only p53 immunohistochemistry was performed, the highest incidence was 3.9%.

Conclusions The characteristics of POLEmut-p53abn resembled those of POLEmut, whereas MMRd-p53abn appeared to be intermediate between MMRd and p53abn. The high proportion of multiple classifiers may be related to the methods used for molecular classification, which included both p53 immunohistochemistry and *TP53* sequencing.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Multiple classifiers are endometrial cancers with more than one molecular feature (*POLE* mutation (POLEmut), MMR deficiency (MMRd), or p53 abnormality (p53abn)). Previous studies reported an incidence of 3–6% of all endometrial cancers. Based on preliminary data from a few retrospective studies, patients with *POLE* mutation are classified as POLEmut even in the presence of MMRd and/or p53 abnormality, while MMRd-p53abn are classified as MMRd.

WHAT THIS STUDY ADDS

⇒ In our prospective cohort, 11% of endometrial cancers were multiple classifiers. This finding, probably due to the thorough molecular analysis that included an assessment of both p53 expression and *TP53* mutations, underscores the critical need to clarify the role of multiple classifiers. MMRd-p53abn had characteristics that appeared to be intermediate between those of MMRd and p53abn, whereas POLEmut-p53abn was similar to POLEmut endometrial cancer.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Collaborative studies worldwide should aim to understand the role of multiple classifiers in predicting outcomes in endometrial cancer. The hierarchical classification approach may hinder an accurate identification of multiple classifiers, which instead requires simultaneous molecular analysis.

INTRODUCTION

The Cancer Genome Atlas Research (TCGA) Network classified endometrial cancers into four categories: DNA polymerase epsilon (*POLE*)/ultramutated, microsatellite instable/hypermutated, copy-number low, and copy-number high.[1] Despite its prognostic significance, the application of this classification was impractical due to the complex and expensive technologies required. To bring it into practice, the TransPORTEC and Vancouver groups proposed

surrogate biomarkers that could mimic the TCGA.[2–7] Accordingly, *POLE* ultramutated can be identified by *POLE* exonuclease domain sequencing, microsatellite instable/hypermutated by mismatch repair protein immunohistochemistry(MMRd), and copy number high by p53 immunohistochemistry(p53abn). Endometrial cancers lacking these features are called 'no specific molecular profile' and resemble the copy-number low group. *POLE* exonuclease domain sequencing identifies all *POLE* ultramutated and MMR immunohistochemistry has a high concordance with the microsatellite instability assay. Although p53 immunostaining/*TP53* mutation status is not equivalent to the copy-number high subgroup, it can identify cases with significantly worse outcomes and its clinical usefulness has already been accepted.[3 8]

Nowadays, guidelines recommend the use of this classification along with traditional histopathologic risk factors to better characterize endometrial cancer,[9–13] and as a way to risk-stratify patients and to inform decisions about post-operative treatment.[9 11 13]

Most tumors are easily classified into one of the four categories due to the presence of only one molecular feature (hereafter referred to as 'single classifiers'). Conversely, some tumors have more than one feature and are called 'multiple classifiers'

In this study, we primarily aimed to describe the clinicopathological and molecular characteristics and the oncologic outcomes of our cohort of multiple classifiers. A secondary objective was to compare the characteristics of multiple classifiers with those of single classifiers that shared at least one molecular feature. Furthermore, we conducted a review of the literature on the incidence of multiple classifiers and the techniques used for molecular classification.

METHODS

Case Selection

From the prospectively collected database of endometrial cancers treated at the European Institute of Oncology, Milan, Italy, we identified consecutive cases with molecular analysis (performed systematically on all endometrial cancers since April 2019). Patients were included if they had undergone surgical staging between April 2019 and December 2022, regardless of histologic and clinical characteristics, and if *POLE*, microsatellite instability/MMR, and p53/*TP53* status were known. Patients not consenting to data use for clinical research or not undergoing surgical staging were excluded. A subset of this cohort has been described in other studies.[14 15]

Molecular Analyses and Classification

POLE and *TP53* mutations were identified by next-generation sequencing using a panel of 26 cancer-related genes. *POLE* mutations (exons 9, 13, 14) were classified according to the literature,[16] whereas *TP53* mutations were evaluated using the COSMIC, ClinVar and cBioPortal Databases. MMR immunohistochemistry was classified as proficient if MSH6, PMS2, MSH2, and MLH1 were expressed, MMRd if at least one protein expression was lost, and equivocal in cases of equivocal staining. The Idylla microsatellite instability assay (Biocartis, Mechelen, Belgium) for seven microsatellite regions (*ACVR2A, BTBD7, DIDO1, MRE11, RYR3, SEC31A, SULF2*) was also performed. In cases of discrepancy between immunohistochemistry and the Idylla assay, the Promega microsatellite instability analysis system (version 1.2) was used. A tumor was classified as MMRd if either MMR proteins were not expressed or microsatellite instability assessment was positive. Immunohistochemistry for p53 was classified as normal/wild type, aberrant (overexpression, null, cytoplasmic), or subclonal according to the literature.[4 17 18] A tumor was considered p53abn if either p53 staining was aberrant or *TP53* gene harbored a pathogenic/probably pathogenic mutation. Cases without any molecular features were considered as no specific molecular profile. The ProMisE and TransPORTEC molecular algorithms omitted microsatellite instability assessment and TP53 sequencing, both of which we performed on all patients.[2 3]

Cases were then classified as single classifiers (POLEmut, MMRd, p53abn, no specific molecular profile) or multiple classifiers (POLEmut-MMRd, POLEmut-p53abn, MMRd-p53abn, POLEmut-MMRd-p53abn).

Clinicopathological Characteristics and Oncologic Outcomes

Age at surgery and body mass index, International Federation of Gynecology and Obstetrics (FIGO) 2009 stage (I–II vs III–IV) and grade (G1–2 vs G3), histotype (endometrioid vs non-endometrioid), myometrial invasion (none vs <50% vs ≥50%), lymphovascular space invasion absent/focal vs diffuse), lymph node metastases (negative vs isolated tumor cells vs micro/macrometastases), risk groups according to the European Societies of Gynecological Oncology, Radiotherapy and Oncology, and Pathology (ESGO/ESTRO/ESP) guidelines, dates of first recurrence, and last follow-up were collected.[9 19 20]

The study was deemed exempt from ethical approval by the European Institute of Oncology ethics committee (UID2418). In accordance with journal guidelines, our data are available for independent analysis in the online supplemental material. The study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.[21]

Statistical Analysis

Differences between categorical variables were assessed using the X^2 or Fisher's exact tests, with Bonferroni correction for multiple comparisons.[22] One-way analysis of variance with Bonferroni correction was used for differences between continuous variables. Multiple pairwise comparisons were performed when a significant difference ($p<0.05$) was found between three subgroups. Median time to recurrence and follow-up and disease-free survival with 95% CI were estimated through the Kaplan-Meier method. A log-rank test was used to compare survival among groups. A p value <0.05 was considered significant. Analyses were performed with Stata Statistical Software: Release 17 (StataCorp. 2021. College Station, Texas, USA: StataCorp LLC).

Literature Review

The PubMed Database was searched. Articles were included if they were published in English in the last 10 years before August 4, 2022, and included endometrial cancer molecular classification. The search strategy was designed by an experienced librarian with input from the study authors. The actual approach, listing all search terms used and how they were combined, is available in the online supplemental appendix. Study screening and data extraction were performed by two independent reviewers (LADV and IB).

RESULTS

Between April 2019 and December 2022, 498 patients were treated at our institution. Among them, 422 met all the eligibility criteria,

including complete molecular analysis: 374 (88.6%) single classifiers and 48 (11.4%) multiple classifiers. Specifically, 22 (5.2%) POLEmut, 99 (23.5%) MMRd, 180 (42.7%) no specific molecular profile, 73 (17.3%) p53abn, 2 (0.5%) POLEmut-MMRd, 15 (3.6%) POLEmut-p53abn, 28 (6.6%) MMRd-p53abn, 3 (0.7%) POLEmut-MMRd-p53abn (online supplemental table 2).

MMRd-p53abn

MMRd-p53abn was the largest group with 28 cases. They were mainly early-stage (60.7%), endometrioid (85.2%), high-grade (55.6%), invading<50% of the myometrium (74.1%), without diffuse lymphovascular space invasion (81.5%), and without nodal involvement (74.1%) (table 1). Based on their histomorphologic features, 53.6% were classified as low or intermediate risk according to ESGO/ESTRO/ESP guidelines.

Comparison of MMRd-p53abn with MMRd and p53abn

Compared with MMRd, MMRd-p53abn were more likely to be non-endometrioid (14.8% vs 2.0%, p=0.006) and high-grade (55.6% vs 22.2%, p=0.001). Compared with p53abn, multiple classifiers were less likely to be non-endometrioid (14.8% vs 50.0%, p=0.001) (table 1).

In terms of molecular characteristics, expression of MMR proteins differed significantly between MMRd-p53abn and MMRd. Multiple classifiers showed a lower proportion of abnormal expression of MLH1 (44.4% vs 71.4%, p=0.010) and PMS2 (44.4% vs 78.4%, p<0.001) and a higher proportion of MSH6 loss (55.6% vs 19.4%, p=0.001). Compared with p53abn endometrial cancer, p53 subclonal pattern was more frequent in MMRd-p53abn (18.2% vs 2.9%, p=0.005). Five (17.9%) MMRd-p53abn had more than one *TP53* mutation, in contrast to only 3 (4.1%) p53abn.

Two (7.1%) recurrences were observed in MMRd-p53abn at 5.0 and 6.9 months after surgery, while MMRd and p53abn recurred in 4 (4.0%) and 25 (34.3%) cases, respectively, with a median time to recurrence of 8.8 and 8.4 months (online supplemental figure 2). Three-year disease-free survival was 87.8% (95% CI 59.6 to 96.8), 91.9% (95% CI 78.7 to 97.0), and 38.0% (95% CI 20.2 to 55.8) for MMRd-p53abn, MMRd, and p53abn, respectively (p<0.001). The median follow-up of patients without recurrences was 4.2, 11.6, and 10.8 months in MMRd-p53abn, MMRd, and p53abn, respectively.

POLEmut-p53abn

POLEmut-p53abn accounted for 15 cases. Most were early-stage (93.3%), endometrioid (93.3%), high-grade (66.7%), invading <50% of the myometrium (66.6%), and without diffuse lymphovascular space invasion (93.3%) or nodal involvement (100.0%) (table 2). Based on histomorphologic features, 66.6% were low or intermediate risk according to ESGO/ESTRO/ESP guidelines.

Comparison of POLEmut-p53abn with POLEmut and p53abn

POLEmut-p53abn differed from p53abn in age at surgery (56.1 vs 66.7 years, p=0.003), advanced-stage (6.7% vs 53.4%, p=0.001), and non-endometrioid histotype (6.7% vs 50.0%, p=0.002), showing similar characteristics to POLEmut tumors. The only exception was grade, as POLEmut-p53abn differed significantly from POLEmut (high-grade: 66.7% vs 22.7%, p=0.008) but not from p53abn (71.2%, p=0.724). Although not significant, most POLEmut and POLEmut-p53abn had no nodal metastases, whereas p53abn had 22.6% positive nodes.

In POLEmut-p53abn, *POLE* variants were 7 (46.7%) P286R, 4 (26.7%) V411L, 2 (13.3%) S297F, 1 (6.7%) S459F, 1 (6.7%) A456P. Similarly, P286R and V411L were the most common in POLEmut, accounting for 14 (63.6%) and 5 (22.7%) cases, respectively. POLEmut-p53abn showed fewer aberrant and more subclonal p53 stainings compared with p53abn endometrial cancers (p<0.001).

No recurrences were observed in 15 POLEmut-p53abn and 22 POLEmut during a median follow-up of 12.8 and 12.6 months, respectively (online supplemental figure 3).

POLEmut-MMRd and POLEmut-MMRd-p53abn

Two POLEmut-MMRd were both endometrioid, stage IA, low-grade, without lymphovascular space invasion. Isolated tumor cells in sentinel lymph nodes were identified in one of them. *POLE* mutations were S297F and P286R.

We identified three early-stage, high-grade, endometrioid or mixed histotypes POLEmut-MMRd-p53abn with six different *TP53* mutations and a unique *POLE* mutation (V411L).

After a median follow-up of 17.0 months, no recurrences were observed in either of these two groups.

TP53 Sequencing and p53 Immunohistochemistry Discordance

TP53 sequencing and p53 immunostaining were both available in 364 (86.3%) cases with conflicting results (ie, one wild-type and the other abnormal) in 44 (12.1%) cases: 32/327 (9.8%) single and 12/37 (32.4%) multiple classifiers (online supplemental table 3). Sequencing allowed the identification of 7 (18.9%) additional cases of multiple classifiers that had normal p53 immunohistochemistry: 3 POLEmut-p53abn and 4 MMRd-p53abn.

Microsatellite Instability Assay and MMR Immunohistochemistry Discordance

Microsatellite instability assay and MMR immunostaining were both available in 395 (93.6%) cases and gave conflicting results (ie, MMRp-microsatellite instable or MMRd-microsatellite stable) in 20 (5.1%) cases: 16/355 (4.5%) single and 4/40 (10.0%) multiple classifiers (online supplemental table 4). MMR immunostaining allowed identification of 4 (10.0%) additional cases of multiple classifiers that were microsatellite stable: 2 MMRd-p53abn, 1 POLEmut-MMRd, and 1 POLEmut-MMRd-p53abn. Microsatellite instability assay did not identify any additional patients with normal MMR protein expression.

Literature Review

The PubMed search returned 192 records. Screening of the reference lists of the included publications identified four additional studies. We excluded 146 records based on titles and abstracts and 34 after full text screening. Ultimately, 16 published records from 15 studies were available for the analysis.[1–6 16 23–31] The study flow diagram is reported in online supplemental figure 1.

All the records were published between 2013 and 2021, after the publication of the TCGA report.[1] Fourteen studies were retrospective, 10 single-center and four multicenter. One study was described as single-center prospective.[29] The largest study by León-Castillo et al[16 23] pooled data from the PORTEC, Vancouver, TCGA, and two independent cohorts.[1 4–6 32 33] Eight groups included

Table 1 Comparison of baseline characteristics between MMRd, p53abn, and MMRd-p53abn

Characteristics	Molecular class			P value	Multiple comparisons p value	
	MMRd (n=99)	p53abn (n=73)	MMRd-p53abn (n=28)	MMRd vs p53abn vs MMRd-p53abn	MMRd vs MMRd-p53abn	p53abn vs MMRd-p53abn
Clinical and pathologic characteristics						
Age at surgery (years), mean (SD)	61.3 (10.2)	66.7 (11.0)	63.0 (10.7)	**<0.001**	1.000	0.353
BMI (kg/m^2), mean (SD)	27.6 (7.8)	28.0 (6.4)	27.8 (5.5)	0.51	–	–
Early vs advanced-stage, n (%)				**0.002**	0.220	0.203
Early (I–II)	72 (72.7)	34 (46.6)	17 (60.7)			
Advanced	27 (27.3)	39 (53.4)	11 (39.3)			
Endometrioid vs other, n (%)				**<0.001**	**0.006**	**0.001**
Endometrioid	97 (98.0)	36 (50.0)	23 (85.2)			
Non-endometrioid	2 (2.0)	36 (50.0)	4 (14.8)			
Missing	0	1	1			
Grade, n (%)				**<0.001**	**0.001**	0.139
G1–2	77 (77.8)	21 (28.8)	12 (44.4)			
G3	22 (22.2)	52 (71.2)	15 (55.6)			
Missing	0	0	1			
Myometrial invasion, n (%)				0.58	–	–
None	14 (14.1)	10 (14.1)	4 (14.8)			
<50	52 (52.5)	31 (43.7)	16 (59.3)			
≥50	33 (33.3)	30 (42.3)	7 (25.9)			
Unknown	0	2	1			
LVSI, n (%)				0.87	–	–
Absent or focal	81 (82.7)	54 (79.4)	22 (81.5)			
Diffuse	17 (17.4)	14 (20.6)	5 (18.5)			
Missing	1	5	1			
Lymph node metastases, n (%)				0.79	–	–
Negative	76 (80.0)	40 (75.5)	20 (74.1)			
ITC	4 (4.2)	1 (1.9)	1 (3.7)			
Micro/macro	15 (15.8)	12 (22.6)	6 (22.2)			
Missing/unknown	4	20	1			
Risk classification*, n (%)				**<0.001**	0.149	0.058
Low	43 (43.4)	10 (13.7)	7 (25.0)			
Intermediate	20 (20.2)	8 (11.0)	8 (28.6)			
High-intermediate	14 (14.1)	8 (11.0)	3 (10.7)			
High	21 (21.2)	30 (41.1)	8 (28.6)			
Advanced/metastatic	1 (1.0)	17 (23.3)	2 (7.1)			
Molecular characteristics						
MMR proteins deficiency						
MLH1, n (%)				–	**0.010**	
Loss of expression	70 (71.4)	–	12 (44.4)			
Equivocal	3 (3.1)	–	0			
Normal expression	25 (25.5)	–	15 (55.6)			
Missing	1		1			
PMS2, n (%)				–	**<0.001**	
Loss of expression	76 (78.4)	–	12 (44.4)			
Equivocal	3 (3.1)	–	0			
Normal expression	18 (18.6)	–	15 (55.6)			

Continued

Table 1 Continued

Characteristics	Molecular class			P value	Multiple comparisons p value	
	MMRd (n=99)	p53abn (n=73)	MMRd-p53abn (n=28)	MMRd vs p53abn vs MMRd-p53abn	MMRd vs MMRd-p53abn	p53abn vs MMRd-p53abn
Missing	2		1			
MSH2, n (%)				–	0.690	
Loss of expression	10 (10.2)	–	4 (14.8)			
Equivocal	2 (2.0)	–	1 (3.7)			
Normal expression	86 (87.8)	–	22 (81.45)			
Missing	1		1			
MSH6, n (%)				–	**<0.001**	
Loss of expression	19 (19.4)	–	15 (55.6)			
Equivocal	3 (3.1)	–	1 (3.7)			
Normal expression	76 (77.6)	–	11 (40.7)			
Missing	1		1			
MSI, n (%)				–	0.560	
MSI	83 (88.3)	–	24 (92.3)			
MSS	11 (11.7)	–	2 (7.7)			
Missing	5		2			
p53 IHC, n (%)				–		0.005
Aberrant	–	64 (91.4)	14 (63.6)			
Subclonal	–	2 (2.9)	4 (18.2)			
Wild type expression	–	4 (5.7)	4 (18.2)			
Missing		3	6			
TP53 NGS, n (%)				–		0.361
Pathogenic	–	67 (91.8)	24 (85.7)			
Wild type	–	6 (8.2)†	4 (14.3)			

*ESGO/ESTRO/ESP guidelines, 2020 – molecular classification unknown.
†A case of VUS was considered as wild-type.
BMI, body mass index; ESGO, European Society of Gynaecological Oncology; ESP, European Society of Pathology; ESTRO, European Society for Radiotherapy and Oncology; IHC, immunohistochemistry; ITC, isolated tumor cells; LVSI, lymphovascular space invasion; MMRd, mismatch repair deficient; MSI, microsatellite instability; MSS, microsatellite stable; NGS, next-generation sequencing; p53abn, p53 abnormal; SD, standard deviation; VUS, variant of uncertain significance.

all comers' endometrial cancers, whereas the remaining analyzed high-risk populations (table 3).

Diagnostic Tests

Included studies performed Sanger or next-generation sequencing (exons 9, 13, 14, or 9–14) for *POLE*. MMR status was assessed by immunohistochemistry only in nine studies, microsatellite instability only in three and a combination of the two techniques in three studies. p53 status was assessed by immunohistochemistry only in seven studies, sequencing only in one and a combination of the two techniques in seven studies.

Incidence of Multiple-Classifiers

The incidence of multiple classifiers ranged from 1.8% to 14.3% (figure 1). In 10 studies with more than one hundred patients, the incidence was between 1.8% and 9.8%.[16] Among the four studies that assessed p53 status by immunohistochemistry alone in more than 100 patients, the highest incidence was 3.9%.[24] Conversely, the incidence was between 2.6% and 9.8% when *TP53* sequencing was performed. The most frequent subgroup was MMRd-p53abn, followed by POLEmut-MMRd, POLEmut-p53abn, and POLEmut-MMRd-p53abn (figure 1 and table 3).

DISCUSSION

Summary of Main Results

In our cohort, approximately 1/10 endometrial cancer was a multiple classifier. The clinicopathological characteristics of MMRd-p53abn were intermediate between those of p53abn and MMRd for histotype, grade, and MMR proteins expression. POLEmut-p53abn resembled POLEmut in clinicopathological, molecular characteristics, and oncologic outcomes. Subclonal p53 expression was more frequent in multiple classifiers than in p53abn single classifiers.

Results in the Context of Published Literature

This is the first attempt to compare clinicopathological and molecular characteristics between single and multiple classifiers in a cohort of consecutive patients. With the exception of León-Castillo

Table 2 Comparison of baseline characteristics between POLEmut, p53abn, and POLEmut-p53abn

Characteristics	Molecular class			P value	Multiple comparisons P value	
	POLEmut (n=22)	p53abn (n=73)	POLEmut-p53abn (n=15)	POLEmut vs p53abn vs POLEmut-p53abn	POLEmut vs POLEmut-p53abn	p53abn vs POLEmut-p53abn
Clinical and pathologic characteristics						
Age at surgery (years), mean (SD)	56.6 (11.0)	66.7 (11.0)	56.1 (10.9)	**<0.001**	1.000	**0.003**
BMI (kg/m^2), mean±SD	26.0 (5.5)	28.0 (6.4)	23.6 (3.7)	0.055	0.796	0.060
Early vs advanced-stage, n (%)				**<0.001**	0.791	**0.001**
Early (I–II)	20 (90.9)	34 (46.6)	14 (93.3)			
Advanced	2 (9.1)	39 (53.4)	1 (6.7)			
Endometrioid vs other, n (%)				**<0.001**	0.220	**0.002**
Endometrioid	22 (100.0)	36 (50.0)	14 (93.3)			
Non-endometrioid	0	36 (50.0)	1 (6.7)			
Missing	0	1	0			
Grade, n (%)				**<0.001**	**0.008**	0.724
G1–2	17 (77.3)	21 (28.8)	5 (33.3)			
G3	5 (22.7)	52 (71.2)	10 (66.7)			
Myometrial invasion, n (%)				0.14	–	–
None	6 (27.3)	10 (14.1)	5 (33.3)			
<50	12 (54.6)	31 (43.7)	5 (33.3)			
≥50	4 (18.2)	30 (42.3)	5 (33.3)			
Unknown	0	2	0			
LVSI, n (%)				0.45	–	–
Absent or focal	18 (81.8)	54 (79.4)	14 (93.3)			
Diffuse	4 (18.2)	14 (20.6)	1 (6.7)			
Missing	0	5	0			
Lymph node metastases, n (%)				0.11	–	–
Negative	21 (95.5)	40 (75.5)	13 (100.0)			
ITC	0	1 (1.9)	0			
Micro/macro	1 (4.6)	12 (22.6)	0			
Missing/unknown	0	20	2			
Risk classification*, n (%)				**<0.001**	0.135	**0.007**
Low	15 (68.2)	10 (13.7)	5 (33.3)			
Intermediate	2 (9.1)	8 (11.0)	5 (33.3)			
High-intermediate	2 (9.09)	8 (11.0)	3 (20.0)			
High	3 (13.6)	30 (41.1)	2 (13.3)			
Advanced/metastatic	0	17 (23.3)	0			
Molecular characteristics						
p53 IHC, n (%)				–		**<0.001**
Aberrant	–	64 (91.4)	5 (41.7)			
Subclonal	–	2 (2.9)	4 (33.3)			
Wild-type expression	–	4 (5.7)	3 (25.0)			
Missing		3	3			
TP53 NGS, n (%)				–		0.840
Pathogenic	–	67 (91.8)	14 (93.3)			
Wild type	–	6 (8.2)†	1 (6.7)			

* ESGO/ESTRO/ESP guidelines,2020 – molecular classification unknown.
†a case of VUS was considered as wild-type.
BMI, body mass index; ESGO, European Society of Gynaecological Oncology; ESP, European Society of Pathology; ESTRO, European Society for Radiotherapy and Oncology; IHC, immunohistochemistry; ITC, isolated tumor cells; LVSI, lymphovascular space invasion; NGS, next-generation sequencing; p53abn, p53 abnormal; POLEmut, POLE mutated; SD, standard deviation; VUS, variant of uncertain significance.

Table 3 Characteristics of the included studies, ordered by number of patients

Study	Design	*POLE* seq	MSI	MMR IHC	*TP53* seq	p53 IHC	Patients (n)	Type of EC	Patient N	Stage I–II n (%)	Endometrioid n (%)	Multiple-classifier n (%)	MMRd-p53abn n (%)	POLEmut-p53abn n (%)	POLE-MMRd n (%)	Triple positive n (%)
León-Castillo, 2020[16 23]	Multi	+	–	+	±	+	3518	All types	3518	NA	NA	137 (3.9)	64 (1.8)	31 (0.9)	30 (0.9)	12 (0.3)
Stelloo, 2016*[5]	Multi	+	+	–	±	+	861	High-intermediate risk	861	861 (100.0)	861 (100.0)	27 (3.1)	13 (1.5)	7 (0.8)	6 (0.7)	1 (0.1)
Kandoth, 2013*[1]	Multi	+	+	–	+	–	530	High risk	530	NA	NA	52 (9.8)	23 (4.3)	13 (2.5)	9 (1.7)	7 (1.3)
Kommoss, 2018*[6]	Single	+	–	+	–	+	452	All types	452	391 (86.5)	397 (87.8)	8 (1.8)	1 (0.2)	2 (0.4)	5 (1.1)	0 (0)
Talhouk, 2017*[4]	Single	+	–	+	+	+	319	All types	319	NA	215 (67.4)	16 (5)	11 (3.4)	4 (1.3)	0 (0)	1 (0.3)
Britton, 2019[24]	Single	+	–	+	–	+	257	All EC <50y.o.	257	NA	225 (87.5)	10 (3.9)	8 (3.1)	0 (0)	2 (0.8)	0 (0)
Talhouk, 2015[3]	Single	+	–	+	+	+	143	All types	143	NA	119 (83.2)	12 (8.4)	9 (6.3)	3 (2.1)	0 (0)	0 (0)
Victoor, 2021[25]	Single	+	±	+	–	+	120	All types	120	95 (79.2)	84 (70.0)	4 (3.3)	0 (0)	1 (0.8)	0 (0)	3 (2.5)
Stelloo, 2015[2]	Multi	+	+	–	±	+	116	High risk	116	63 (54.3)	86 (74.1)	3 (2.6)	1 (0.9)	0 (0)	2 (1.7)	0 (0)
Timmerman, 2020[26]	Single	+	±	+	–	+	108	All types	108	84 (77.8)	83 (76.9)	4 (3.7)	0 (0)	1 (0.9)	3 (2.8)	0 (0)
Joehlin-Price, 2021[27]	Single	+	–	+	–	+	95	G3 endometrioid	95	61 (64.2)	95 (100.0)	6 (6.3)	1 (1.1)	1 (1.1)	3 (3.2)	1 (1.1)
Kim, 2020[28]	Single	+	–	+	–	–	52	Clear cell	52	35 (67.3)	0	2 (3.8)	2 (3.8)	0 (0)	0 (0)	0 (0)
Knez, 2021†[29]	Single	+	–	+	–	+	45	All types	45	34 (75.6)	41 (91.1)	6 (13.3)	2 (4.4)	4 (8.9)	0 (0)	0 (0)
Kobayashi, 2021[30]	Single	+	+	+	+	+	36	High risk	36	NA	19 (52.8)	5 (13.9)	4 (11.1)	1 (2.8)	0 (0)	0 (0)
Zhang, 2021[31]	Single	+	–	+	–	+	21	Undifferentiated or dedifferentiated	21	13 (61.9)	0	3 (14.3)	0 (0)	1 (4.8)	2 (9.5)	0 (0)
Current study	Single	+	+	+	+	+	422	All types	422	292 (69.2)	370 (87.7)	48 (11.4)	28 (6.6)	15 (3.6)	2 (0.5)	3 (0.7)

The most frequent multiple-classifier class per study is highlighted in green.
*Patients included in the study by León-Castillo et al.[16]
†Prospective study; all the remaining studies were retrospective.
EC, endometrial cancer; IHC, immunohistochemistry; MMR, mismatch repair; MMRd, mismatch repair deficient; MSI, microsatellite instability; multi, multicenter; NA, not available; p53abn, p53 abnormal; POLEmut, POLE mutated; seq, sequencing; single, single center, triple positive.

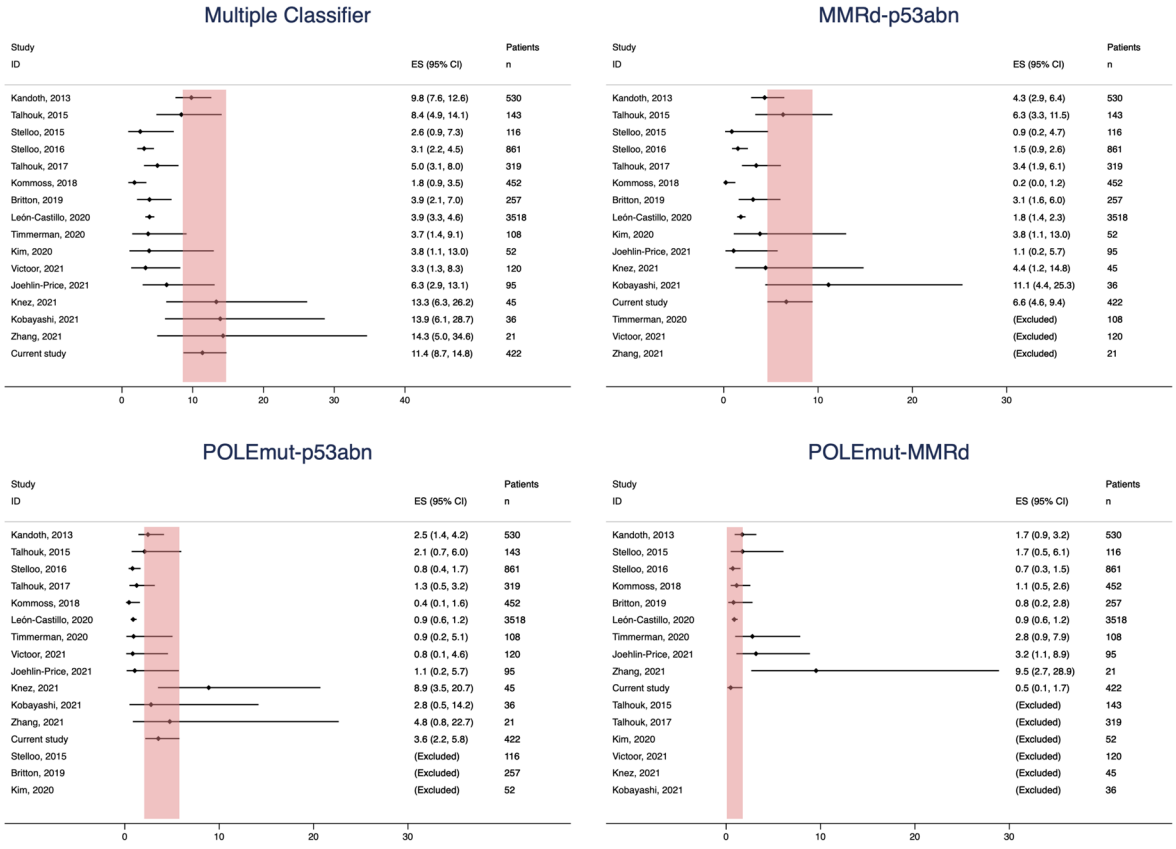

Figure 1 Forest plot of the proportion of multiple classifiers, MMRd-p53abn, POLEmut-p53abn, and POLEmut-MMRd, according to the literature. The red bar represents the CI of the current study. ES, effect-size estimate with 95% CI; MMRd, mismatch repair deficient; N, number; POLEmut, POLE mutated; p53abn, p53 abnormal.

et al, who analyzed 137 multiple classifiers and will be the focus of our discussion, other studies identified only 12 or fewer cases.[16 23]

León-Castillo et al described 64 MMRd-p53abn as predominantly endometrioid, high-grade, early-stage, without lymphovascular space invasion, and with a high proportion of MSH6±MSH2 or single PMS2 loss. They also revealed a higher frequency of multiple *TP53* mutations and subclonal p53abn staining compared with single-classifier p53abn. For genomic features and survival outcomes, MMRd-p53abn was more similar to single-classifier MMRd than to p53abn. The 5-year recurrence-free survival of stage I MMRd-p53abn was significantly different from that of p53abn (92.2% vs 70.8%). However, they did not compare MMRd-p53abn survival with MMRd. These findings are consistent with our results, but we also found a difference in grade and histology between MMRd-p53abn and MMRd, which should be further analyzed in subsequent studies.

Our results on POLEmut-p53abn are in agreement with those of Leon-Castillo et al. They described 31 cases as mostly endometrioid, early-stage, high-grade, without lymphovascular space invasion, and characterized by p53 subclonality and multiple *TP53* mutations. POLEmut-p53abn clustered mainly with POLEmut rather than p53abn. Accordingly, 5-year recurrence-free survival was significantly different from p53abn when the analysis was limited to stage I disease (94.1% vs 70.8%). Similarly, our subset of POLEmut-p53abn showed an excellent prognosis and a high similarity to POLEmut in terms of clinicopathological and molecular characteristics.

Leon-Castillo et al also described 14 POLEmut-MMRd with a probably pathogenic *POLE* mutation, which had a 5-year recurrence-free survival of 92.3%. In addition, 12 endometrial cancers belonged to POLEmut-MMRd-p53abn, which were predominantly early-stage endometrioid, but two were classified as mixed histology. All patients showed p53 subclonality, and most cases clustered with POLEmut. In our cohort only two POLEmut-MMRd and three POLEmut-MMRd-p53abn were found. Therefore, we cannot draw any conclusions about these rare groups.

The high frequency of multiple classifiers found in our cohort is probably the result of the methodology used for molecular analyses.[34] The combination of *TP53* sequencing and p53 staining has increased the detection of multiple classifiers with p53 abnormalities, as occurred in other studies included in the review (figure 1 and table 3). Indeed, in our study, sequencing allowed the detection of an additional 18.9% of multiple classifiers that had a normal immunohistochemistry for p53.

TP53 mutation is probably a non-driver mutation in multiple classifier, caused by the *POLE* mutation and its ultramutated phenotype.[17] In fact, one in three POLEmut-p53abn showed subclonal p53 expression, potentially indicating a later occurrence of *TP53* mutation in tumor development, unable to affect the immunohistochemical pattern ubiquitously and resulting in a subclonal

pattern.[17 18] Similarly, *TP53* mutations found in MMRd-p53abn are probably non-driver. However, based on our results, *TP53* mutations seemed to influence the tumor characteristics of MMRd-p53abn more than those of POLEmut-p53abn. Furthermore, discordance between p53 immunohistochemistry and *TP53* mutations is higher in multiple (32.4%) than in single classifiers (9.8%). Our findings are consistent with the results of Vermij et al who reported that 22/32 cases with discordance between p53 immunohistochemistry and *TP53* gene status were either POLEmut or MMRd.[18]

Although a subclonal mutation is one of the possible causes of discordance between *TP53* sequencing and p53 immunostaining, we acknowledge additional different explanations, such as mutations in non-sequenced exons, post-translational mechanism independent of the *TP53* gene sequence, misinterpretation by the operator.[35]

Strengths and Weaknesses

This is one of the largest datasets on multiple classifiers based on consecutive patients, making the estimation of incidence reliable. The use of next-generation sequencing and microsatellite instability testing, combined with staining for p53 and MMR protein, is also a strength of the study, as it allowed the detection of otherwise missed multiple classifiers. However, further studies are needed to define the usefulness of this approach, as there is no strong evidence of its prognostic impact. The limited number of cases and short follow-up are obvious limitations (the last patients were treated in December 2022). Hence, survival data should be interpreted with caution.

Implications for Practice and Future Research

Hierarchical molecular algorithms, meaning that testing is stopped as soon as an abnormality is detected, prevents the identification of multiple classifiers. In addition, the use of the original ProMisE algorithm, which starts with the assessment of the MMR protein, hinders the identification of POLEmut-MMRd. Alternatively, to identify all multiple classifiers, a simultaneous approach performing all tests should be used. The algorithm proposed by our group in a previous work can help to reduce the cost of the simultaneous approach without missing multiple classifiers, at least in early-stage endometrial cancer.[15]

Evidence on Lynch syndrome in multiple classifiers is currently limited. Hence, screening should be recommended in MMRd patients, regardless of other molecular features.

Collaborative studies engaging research teams from around the world still need to address the prognostic role of multiple classifiers, since current evidence is based on a small number of patients.

Although the combined approach of *TP53* sequencing/p53 immunohistochemistry may be highly informative from a scientific point of view, further evidence is needed to support its worldwide applicability. Given the routine inclusion of *TP53* gene in a sequencing panel for *POLE* analysis, it is crucial to understand how to manage *TP53* mutant endometrial cancers expressing a normal p53 protein.

CONCLUSIONS

Multiple classifiers accounted for 11% of our cohort. Compared with previous studies, the higher proportion of multiple classifiers may be related to the extensive molecular analysis we performed, which included evaluation of both p53 expression and *TP53* mutations. The characteristics of MMRd-p53abn appeared to be intermediate between those of MMRd and p53abn for histotype, grade, and MMR proteins expression, whereas the characteristics of POLEmut-p53abn were similar to POLEmut.

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