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Research Article

Molecular and Clinicopathologic Characterization of Mismatch Repair-Deficient Endometrial Carcinoma Not Related to *MLH1* Promoter Hypermethylation

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ABSTRACT

Universal tumor screening in endometrial carcinoma (EC) is increasingly adopted to identify individuals at risk of Lynch syndrome (LS). These cases involve mismatch repair-deficient (MMRd) EC without *MLH1* promoter hypermethylation (PHM). LS is confirmed through the identification of germline MMR pathogenic variants (PV). In cases where these are not detected, emerging evidence highlights the significance of double-somatic MMR gene alterations as a sporadic cause of MMRd, alongside *POLE*/*POLD1* exonuclease domain (EDM) PV leading to secondary MMR PV. Our understanding of the incidence of different MMRd EC origins not related to *MLH1*-PHM, their associations with clinicopathologic characteristics, and the prognostic implications remains limited.

In a combined analysis of the PORTEC-1, -2, and -3 trials (n = 1254), 84 MMRd EC not related to *MLH1*-PHM were identified that successfully underwent paired tumor–normal tissue next-generation sequencing of the MMR and *POLE*/*POLD1* genes. Among these, 37% were LS associated (LS-MMRd EC), 38% were due to double-somatic hits (DS-MMRd EC), and 25% remained unexplained. LS-MMRd EC exhibited higher rates of MSH6 (52% vs 19%) or PMS2 loss (29% vs 3%) than DS-MMRd EC, and exclusively showed MMR-deficient gland foci. DS-MMRd EC had higher rates of combined MSH2/MSH6 loss (47% vs 16%), loss of >2 MMR proteins (16% vs 3%), and somatic *POLE*-EDM PV (25% vs 3%) than LS-MMRd EC. Clinicopathologic characteristics, including age at tumor onset and prognosis, did not differ among the various groups.

Our study validates the use of paired tumor–normal next-generation sequencing to identify definitive sporadic causes in MMRd EC unrelated to *MLH1*-PHM. MMR immunohistochemistry and *POLE*-EDM mutation status can aid in the differentiation between LS-MMRd EC and DS-MMRd EC.

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These findings emphasize the need for integrating tumor sequencing into LS diagnostics, along with clear interpretation guidelines, to improve clinical management. Although not impacting prognosis, confirmation of DS-MMRd EC may release patients and relatives from burdensome LS surveillance.

Introduction

Mismatch repair-deficient endometrial carcinomas (MMRd EC) constitute approximately 30% of all endometrial carcinomas (EC).[1] MMRd EC are characterized by deficiencies in the DNA mismatch repair (MMR) system, resulting in the accumulation of insertions and deletions at microsatellites, often leading to microsatellite instability (MSI) and a high tumor mutational burden.[2] MMRd EC arises when 1 of the 4 MMR genes loses its function: mutL homolog 1 (*MLH1*), postmeiotic segregation increased 2 (*PMS2*), mutS homolog 2 (*MSH2*), or mutS homolog 6 (*MSH6*). Approximately 70% of MMRd EC are attributed to acquired promoter hypermethylation of *MLH1* (*MLH1*-PHM), leading to its epigenetic silencing.[3-6] Approximately 10% of MMRd EC are due to Lynch syndrome (LS), a hereditary cancer predisposition syndrome, characterized by germline pathogenic variants (PV) in MMR genes or, rarely, the *EPCAM* gene, which causes epigenetic silencing of the downstream *MSH2* gene.[3,6,7]

Diagnosing LS in cancer-affected individuals is crucial as it allows for initiating cancer surveillance, cancer risk reduction strategies, and genetic cascade testing for at-risk relatives.[8] To detect those at risk of LS, universal screening of all individuals with newly diagnosed EC (independent of age) is increasingly adopted worldwide, as recommended by various international organizations.[9-12] In this approach, EC are tested for MMR deficiency by immunohistochemistry (IHC) of the 4 MMR proteins (MLH1, PMS2, MSH2, and MSH6). Subsequently, *MLH1* promoter methylation assay is conducted in cases of combined MLH1/PMS2 loss. If *MLH1*-PHM is detected, LS is considered to be ruled out. In cases with no evidence of *MLH1*-PHM, combined loss of MSH2/MSH6, or isolated loss of MSH6 or PMS2, individuals are referred to a clinical geneticist for genetic counseling and genetic testing for LS.

Among the patients referred for LS evaluation, it has been reported that approximately 30% to 50% exhibit a germline PV in an MMR gene, thus confirming LS.[3,6,13-16] Excluding these LS-associated cases, it is expected, primarily based on data from colorectal carcinoma (CRC), that roughly half can be categorized as sporadic, resulting from either double-somatic MMR PV or a single-somatic MMR PV with loss of heterozygosity (LOH) of the wild-type allele.[17-26] The remaining half of cases have either one or no somatic alteration in an MMR gene, leaving the etiology of MMR deficiency unresolved. This condition is also referred to as unexplained MMR deficiency.[21] Unexplained MMRd EC encompass a diverse spectrum of tumors, with possible explanations, including previously undetected germline PV in MMR genes due to technical limitations of standard tests, such as Sanger sequencing.[24,25,27,28] Additionally, PV outside of the MMR genes may impact the DNA MMR system. Previous studies investigating the mechanisms underlying unexplained MMRd tumors, primarily focused on MMRd CRC, have reported germline and somatic PV within the exonuclease domains (EDM) of *POLE/POLD1*, leading to subsequent mutations in MMR genes secondary to the ultra-mutated phenotype.[21,29-32] Furthermore, inaccuracies in the diagnosis of unexplained MMRd EC may arise from false-positive MMR-IHC results.[17,21,24]

The current genetic testing approach to determine the risk of LS and guide future management of patients with MMRd EC and their at-risk relatives involves germline testing with peripheral blood DNA. However, considerable heterogeneity exists in the implementation of tumor sequencing, whether conducted sequentially after negative germline testing or as a paired upfront procedure.[33] Omitting tumor sequencing in the genetic risk assessment of LS may result in undetected double-somatic MMR gene alterations, potentially leading to a higher proportion of cases misclassified as unexplained MMRd.[27] Managing patients with unexplained MMRd EC and their at-risk relatives poses clinical challenges due to limited information about the tumor's hereditary or sporadic origins, leading to inaccurate risk assessments and surveillance recommendations, significantly impacting their quality of life.[34-36] This clinical practice heterogeneity partly arises from a lack of consensus in international LS guidelines regarding the use of tumor–normal sequencing to identify sporadic causes and limited guidance for interpreting its results.[33,37] Existing evidence on the prevalence, clinicopathologic features, and prognosis of hereditary and sporadic causes of MMRd EC not related to *MLH1*-PHM originates from limited and often small-sized studies.[17,19,21,22,24-26,34,38,39] Further research is essential to provide insights into differentiating features between hereditary and sporadic cases, which can aid in LS risk assessment and the development of tailored surveillance recommendations. Moreover, there is need for more investigation into the prevalence of *POLE*/*POLD1*-EDM PV across different origins of MMRd EC. According to the ESGO/ESTRO/ESP guidelines, molecular classification is recommended for all newly diagnosed EC, wherein those with a somatic *POLE*-EDM PV, irrespective of their MMR status, are classified as *POLE*-mutant EC.[40] However, the implications of this classification on LS risk estimation and surveillance recommendations lack clarity and are yet to be defined in LS guidelines.

Our study aimed to identify the underlying causes of MMRd EC not related to *MLH1*-PHM in a large combined clinical trial cohort. Additionally, we assessed clinicopathologic characteristics and prognosis associated with the identified underlying causes, providing valuable insights for the diagnostic and clinical guidance of these cases.

Materials and Methods

Cohort Description

A total of 1279 of the 1801 cases in the PORTEC-1, -2, and -3 clinical trials had sufficient tumor material for MMR-IHC. Among these, in 1254 successful MMR-IHC staining results were reported, meeting the criteria for eligibility in the analysis. The design and results of these trials have been published previously.[41-43] In short, the PORTEC-1 trial, conducted from 1990-1997 in The Netherlands, involved 714 patients with early-stage,

intermediate-risk endometrial cancer.[41] The PORTEC-2 trial, conducted from 2002 to 2006 in The Netherlands, included 427 patients with early-stage, high- to intermediate-risk endometrial cancer.[42] The PORTEC-3 trial included 660 patients with stage I to III, high-risk endometrial cancer, conducted in multiple countries, including The Netherlands, United Kingdom, France, Italy, Canada, Australia, and New Zealand.[43] In these trials, individuals with a history of invasive cancer, except for nonmelanoma skin cancer (within the last 10 years for PORTEC-3), were excluded.

Ethics

The study protocols received approval from the Dutch Cancer Society and the medical ethics committees at participating centers. All patients provided informed consent for their trial participation and the use of their tumor samples for subsequent translational research. Specific ethics approval was obtained for variant analysis on normal tissue for individuals suspected of having LS, as previously discussed.[3]

Mismatch Repair Analysis

Tumors underwent screening for MMR deficiency through IHC analysis of the MMR proteins MLH1 (clone ES05, 1:100; DAKO), MSH2 (clone FE11, 1:200, DAKO), MSH6 (clone EPR3945, 1:800, Genetex), and PMS2 (clone EP51, 1:75, DAKO) using whole-slide staining, as previously described.[3,44-46] Tumors were classified as MMRd if they showed complete loss of nuclear staining of at least 1 of the 4 MMR proteins with positive internal control, or subclonal loss (>10%), as previously reported.[3,44-47] Methylation-specific PCR was performed, as previously described, on tumors with MLH1 loss by IHC to analyze *MLH1*-PHM.[48] Cases with combined MLH1/PMS2 loss without *MLH1*-PHM, combined loss of MSH2/MSH6, or isolated loss of MSH6 or PMS2 were selected for paired tumor and tumor-adjacent normal tissue (myometrium) next-generation sequencing (NGS). Cases with successful paired tumor−normal tissue NGS were included in the final study cohort. These included cases were re-reviewed by an expert gynecopathologist (T.B.) for MMR-IHC staining results and the presence of "mismatch repair-deficient-gland foci (MMR-DGF)" in adjacent normal endometrium, which are defined as foci of loss of MMR protein expression in adjacent benign endometrial glands.[49]

Targeted Next-Generation Sequencing

Tumor areas displaying MMR-IHC loss were identified. Tumor DNA and adjacent normal DNA from the myometrium were obtained from formalin-fixed paraffin-embedded (FFPE) tissue, as previously reported.[50] NGS was performed using a custom-designed Ampliseq panel optimized for FFPE, covering the exonic regions of *MLH1*, *MSH2*, *MSH6*, *PMS2*, *POLE,* and *POLD1*. Targeted NGS libraries were prepared with Ion AmpliSeq Library Kit 2.0 according to the manufacturer's protocol (ThermoFisher Scientific). Sequencing was performed on the Ion S5 System by GenomeScan, after loading the samples on Ion Torrent 540 chips using the Ion Chef (Thermo Fisher Scientific). The unaligned bam files generated were mapped against the human reference genome (GRCh37/hg19) using the TMAP software with default parameters (https://github.com/iontorrent/TS).

Subsequently, variant calling was done using the Ion Torrent-specific caller, Torrent Variant Caller. The full data set was filtered and prioritized by variant frequency (>10%) and coverage (>50 times). Integrative Genomics Viewer was used for visually inspecting variants.[51] Variants were filtered, classified, and registered using Geneticist Assistant (Softgenetics LLC), which assigns functional prediction, conservation scores, and disease-associated information to each variant. Likely pathogenic and pathogenic variants were classified as class 4 and 5, respectively. Variants were annotated to the following GenBank reference sequences: NM_000249.3 (*MLH1*), NM_000251.2 (*MSH2*), NM_000179.2 (*MSH6*), NM_000535.5 (*PMS2*), NM_006231.2 (*POLE*), and NM_001256849.1 (*POLD1*). Unknown variants were further studied using Alamut Visual Plus (Softgenetics), and online available databases and tools: InSiGHT variants databases for MMR genes (https://www.insight-group.org), Leiden Open Variation Database (LOVD), Franklin (https://franklin.genoox.com/), The Clinical Knowledgebase (CKB) (https://ckbhome.jax.org/), and genome nexus (https://www.genomenexus.org/).[52]

All patients with MMR germline variants (likely) affecting function were verified by a genetic analyst (C.T.).

Once a pathogenicity classification was assigned to a variant in Geneticist Assistant, the same pathogenicity was automatically attributed the next time the variant was observed. LOH of the MMR genes in the tumor was determined based on the variant allele frequency (VAF) of the variant in the MMR gene and on the VAF of the available SNPs on the MMR by comparing the VAF between the tumor and paired normal DNA samples.

Classification Criteria

Following successful paired tumor−normal tissue NGS, cases were categorized according to the presence of class 4 and 5 (likely) PV—referred to collectively as PV—and somatic LOH in MMR genes that matched the observed loss of MMR protein expression in IHC. These categories were as follows:

1. Lynch syndrome−associated MMRd EC (LS-MMRd): MMRd EC with a germline PV in an MMR gene and a second somatic hit (PV and/or LOH) in the opposite allele of the same MMR gene.

Cases lacking an identifiable germline PV in an MMR gene were categorized according to the results obtained from tumor sequencing as follows:

2. Double-somatic MMRd EC (DS-MMRd): MMRd EC with a somatic PV in an MMR gene and a second somatic hit (PV and/or LOH) in the opposite allele of the same MMR gene.
3. Unexplained MMRd EC (U-MMRd): MMRd EC with either a single-somatic hit (PV or LOH) or no detected somatic hit in an MMR gene.

Microsatellite Instability-PCR and Multiplex Ligation-Dependent Probe Amplification

Included cases were analyzed for MSI after DNA isolation using the Promega MSI analysis system (version 1.2), as previously described.[44,45] Subsequently, U-MMRd EC cases with sufficient DNA available were analyzed for promoter methylation and copy number variation in *MSH2*, *MSH6*, *PMS2*, and *EPCAM*. This analysis was done using the SALSA multiplex ligation-dependent probe amplification (MS-MLPA) probemix ME011 mismatch repair genes, following the instructions provided by the manufacturer

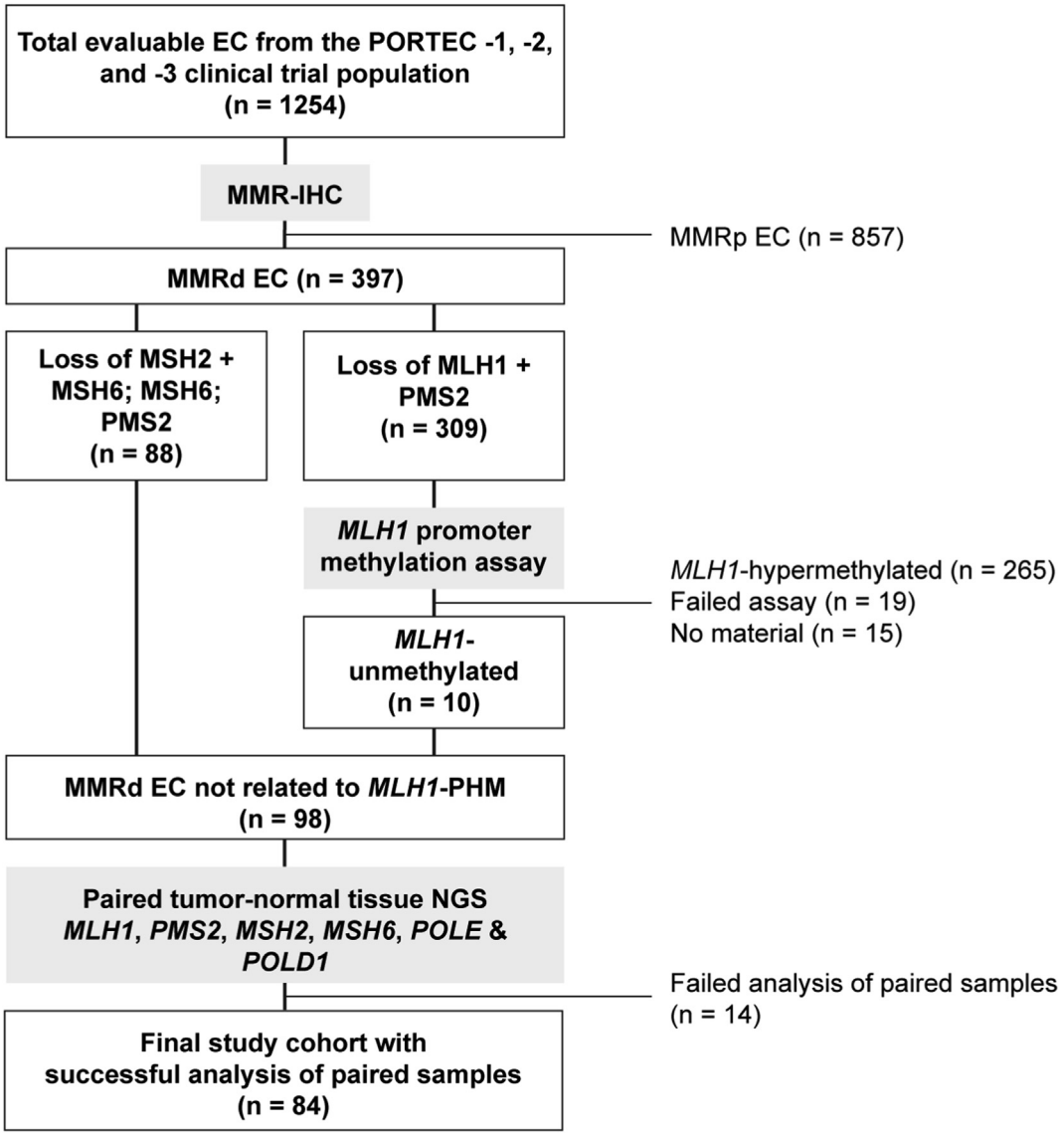

Figure 1.
Flowchart of final study cohort. EC, endometrial carcinomas; MMR-IHC, mismatch repair–immunohistochemistry; MMRd, mismatch repair-deficient; MMRp, mismatch repair-proficient; NGS, next-generation sequencing; PHM, promoter hypermethylation; PORTEC, postoperative radiation therapy in endometrial carcinoma.

(MRC Holland). Copy number variation analysis to detect deletions and duplications was performed using Coffalyser.net (MRC Holland).

Statistical Analysis

Clinical data were extracted from the trial databases for all included patients, and a descriptive analysis was conducted to compare the clinicopathologic characteristics of patients based on the underlying cause of their MMRd EC. Recurrence-free survival (RFS) and overall survival (OS) were estimated using the Kaplan–Meier method and compared with the log-rank test. RFS was defined as the time from random assignment to the first relapse or any cause of death, whichever happened first. OS was the time from random assignment to death from any cause. Patients without an RFS or OS event were marked as censored, with their data recorded until their last contact date. The reverse Kaplan–Meier method was used to estimate the median follow-up time. Statistical analyses were performed using SPSS statistics, version 26 (IBM) and R, version 3.6.1. *P* values of <.05 based on 2-sided tests were considered statistically significant.

Results

Study Population

Of the 1254 evaluable cases, 397 were classified as MMRd EC based on MMR-IHC. Among these, 98 MMRd EC cases were identified that were not related to *MLH1*-PHM. These cases demonstrated either combined MLH1/PMS2 loss with no evidence of *MLH1*-PHM, combined loss of MSH2/MSH6, or isolated loss of MSH6 or PMS2 (see Fig. 1). Subsequently, paired tumor–normal tissue NGS was performed on these 98 cases. In 14% of these cases (n = 14/98), paired tumor–normal tissue NGS was

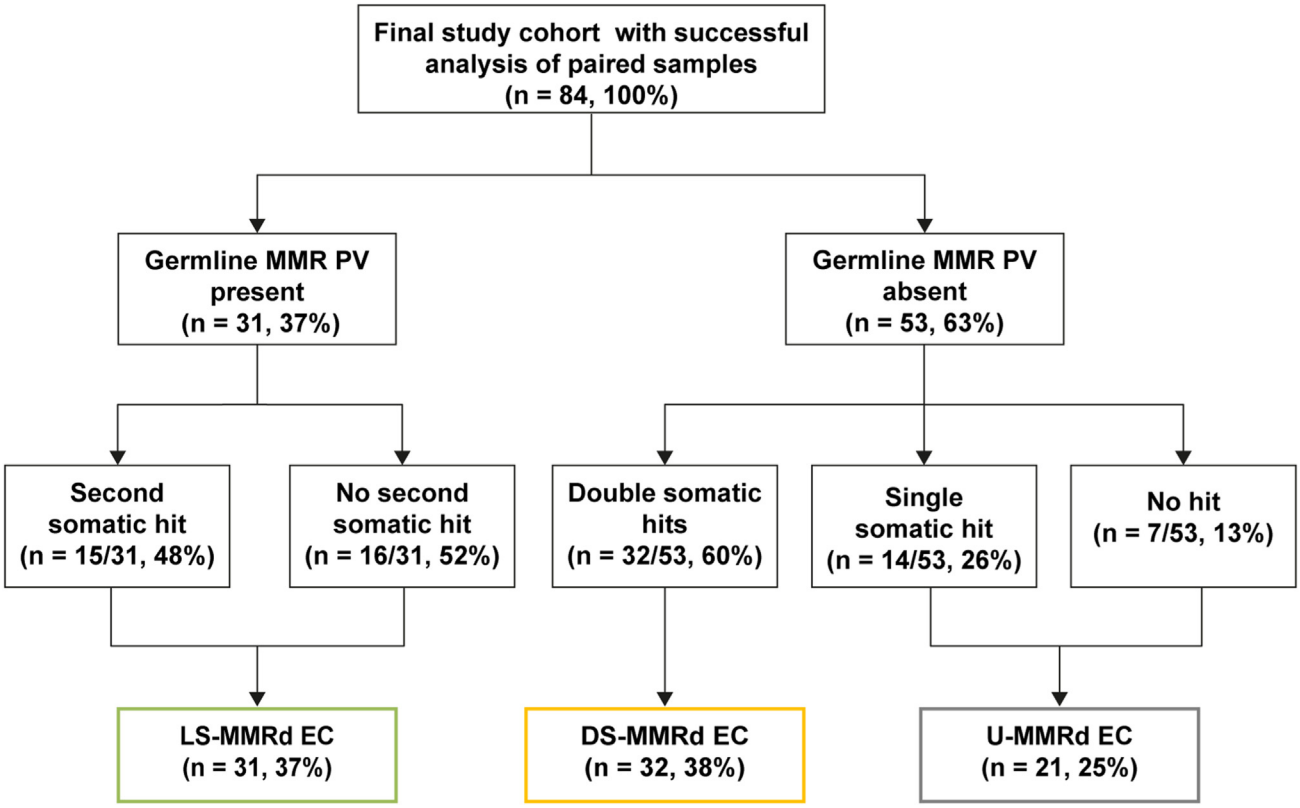

Figure 2.
Summary of results and strategy for detecting the underlying cause of MMR deficiency in the final study cohort, following successful targeted NGS analysis of paired tumor–normal tissue samples. DS, double somatic; EC, endometrial carcinoma; LS, Lynch syndrome; MMRd, mismatch repair-deficient; PV, pathogenic variant; U-MMRd, unexplained mismatch repair-deficient.

unsuccessful due to low DNA yield, or failure in template/library preparation and were subsequently excluded from the study. These failed cases mainly consisted of older samples (ranging between 10 and 32 years). As a result, a total of 84 cases with successful analysis of paired samples were included in the final study cohort.

Mismatch Repair Variants

Of the 84 cases, 31 (37%) had a germline PV in an MMR gene, all of which were consistent with the MMR-IHC results. These cases were classified as LS-MMRd EC, as shown in Figure 2 and Supplementary Table S1. Within these cases, 48% (n = 15/31) exhibited a second somatic hit, either in the form of a somatic PV (n = 11/15), LOH of the wild-type allele (n = 2/15), or a combination of both (n = 2/15).

The remaining 16 LS-MMRd EC cases (52%) did not show evidence of a second somatic hit. Regarding the distribution of germline PV among LS-MMRd EC, the majority was identified in *MSH6* (n = 17/31, 55%), followed by *PMS2* (n = 10/31, 32%) and *MSH2* (n = 4/31, 13%).

Among the 53 cases without a germline PV, 60% (n = 32/53) exhibited double-somatic hits in MMR genes, which matched with the observed protein loss in MMR-IHC. These cases, classified as DS-MMRd EC, had an overall prevalence of 38% (n = 32/84) within the study cohort (Fig. 2). Double-somatic hits were observed as either double-somatic PV (n = 21/32) or as somatic PV accompanied by LOH of the wild-type allele (n = 11/32) (Supplementary Table S2). Regarding the distribution of somatic PV among DS-MMRd EC, the highest proportion was observed in *MSH2* (17/32, 53%), followed by *MSH6* (9/32, 28%), *MLH1* (4/32, 13%), and *PMS2* (2/32, 6%). Furthermore, in 14 of 53 cases without a germline PV (26%), a single-somatic hit was identified, which was concordant with MMR-IHC (Fig. 2). Among these single-hit cases, the majority (n = 9/14) exhibited a single-somatic PV, either in *MSH2* (n = 6/9) or *MSH6* (n = 3/9), and 5 cases displayed somatic LOH of an MMR gene, concordant with MMR-IHC. Among these 5 cases with somatic LOH, 3 cases had a somatic variant of unknown significance in the opposite allele, specifically, *MSH2* (c.809T>G, case #69), *MSH6* (c.2909G>T, case #71), and *PMS2* (c.2285C>T, case #84) as outlined in Supplementary Table S2. In the remaining 13% of cases without germline PV (n = 7/53), there was no evidence of somatic hits in MMR genes. Among these no-hit cases, LOH assessment of the MMR genes was inconclusive in 4 of 7 (Supplementary Table S2).

Of the total single-hit and no-hit cases (n = 21), 10 had DNA available for MS-MLPA targeting *PMS2*, *MSH2*, *MSH6*, and *EPCAM*. However, no deletions or duplications, or aberrant methylation were detected in these genes (Supplementary Table S2). Consequently, these 21 cases were classified as U-MMRd EC, resulting in an overall prevalence of 25% within the study cohort (Fig. 2).

POLE Variants

Overall, 12% of the included cases (n = 10/84) exhibited a somatic *POLE*-EDM PV, with 8 cases observed in DS-MMRd EC.

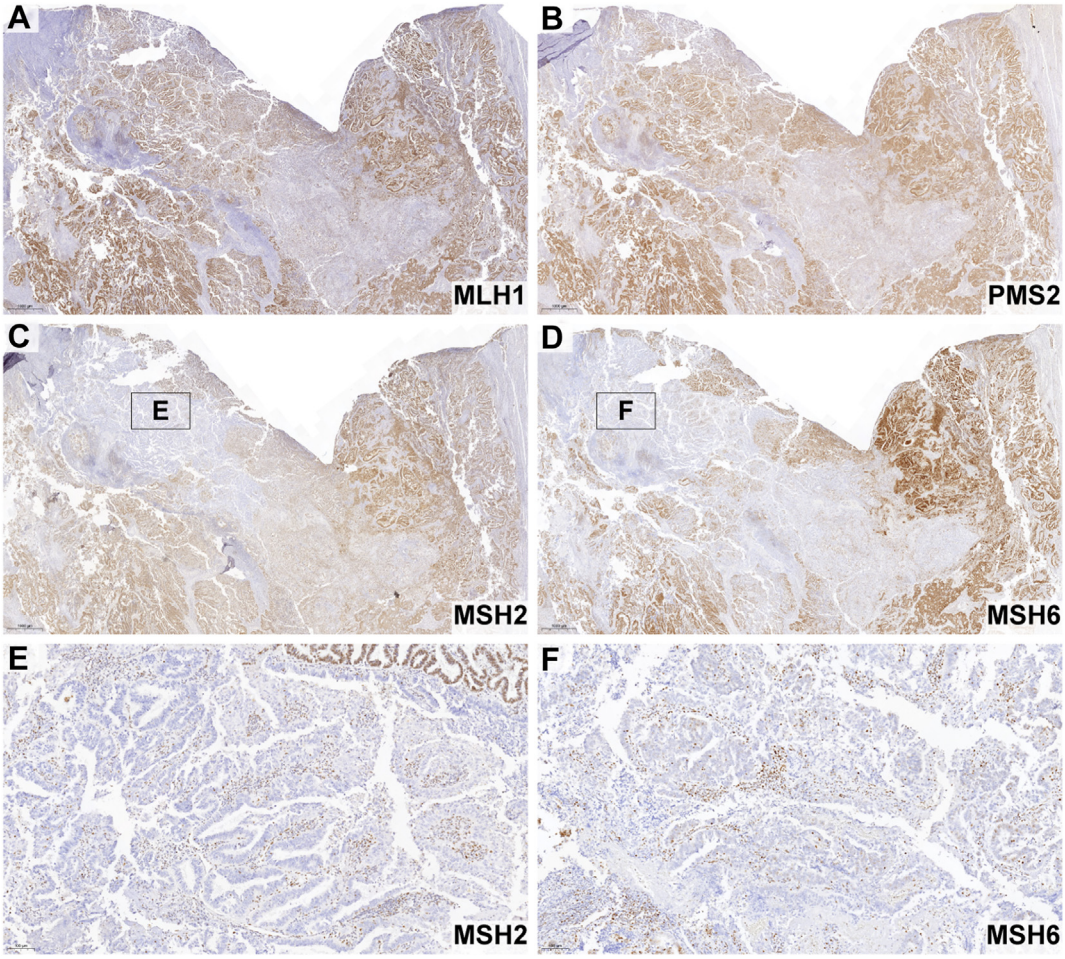

Figure 3.
Representative DS-MMRd EC case (#57) with a somatic *POLE*-EDM pathogenic variant, showing subclonal loss of MSH2 and MSH6 due to double-somatic hits in *MSH2*, whereas MLH1 and PMS2 are retained. ×1 magnification (A-D) ×10 magnification (E. F).

Specifically, these 8 cases had concurrent somatic PV in *MSH6* (n = 4/8), *MSH2* (n = 3/8), and *MLH1* (n = 1/8) (refer to Fig. 3 for a representative image and Supplementary Table S2). The remaining 2 of 10 cases with somatic *POLE*-EDM PV included one in which the *POLE*-EDM PV was detected in an LS-MMRd EC with a germline PV in *PMS2* without a second somatic hit (Supplementary Table S1, case #28). In the other case, both a concurrent somatic *POLE*-EDM PV and somatic LOH of *PMS2* were observed, along with a somatic variant of uncertain significance in *PMS2*, strongly suggesting a sporadic origin (Supplementary Table S2, case #84). No germline *POLE*-EDM PV or any *POLD1*-EDM PV were identified.

Concordance of Mismatch Repair-Deficient and High Microsatellite Instability

In the total cohort, the concordance rate between MMRd and MSI-H was 76% (n = 64/84), with rates of 77%, 81%, and 67% in LS-MMRd EC, DS-MMRd EC, and U-MMRd EC, respectively (Table). Among the discordant cases characterized as MSS or MSI-L (n = 17/84), the most common alteration was MSH6 loss (n = 9/17, 53%), observed in 67% of LS-MMRd EC (n = 4/6), 40% of DS-MMRd EC (n = 2/5), and 50% of U-MMRd EC (n = 3/6). The next most frequent alteration linked to discordant cases involved the subclonal loss of MSH2 and MSH6 (n = 3/17, 18%), with a prevalence of 40% in DS-MMRd EC (n = 2/5) and 17% in U-MMRd EC (n = 1/6). Subsequent MMR-IHC re-evaluation confirmed deficient MMR-IHC status in all MSS/MSI-L cases (refer to Supplementary Figure S1 for a representative image).

Clinicopathologic Characteristics

The clinicopathologic characteristics of the 84 cases, categorized according to their underlying cause of MMRd, are presented in the Table and Figure 4. When comparing LS-associated and DS-MMRd EC cases, no differences were found in age, tumor stage, histologic subtype and grade, presence of lymph-vascular space invasion, and myometrial invasion. A notable distinction was observed in the MMR-IHC expression patterns (Fig. 4). DS-MMRd EC more frequently exhibited combined loss of MSH2/MSH6 compared with LS-MMRd EC (47% vs 16%, respectively). In contrast, LS-MMRd EC was more likely to show isolated loss of MSH6 (52% vs 19%) or PMS2 (29% vs 3%) compared with DS-MMRd EC, respectively. Furthermore, MMR-DGF, involving single glands or clusters of normal endometrial glands with loss of MMR protein expression, was observed in 7% of the cohort (n = 6/84), and it was exclusive to LS-MMRd EC. All these cases were associated with a germline PV in *MSH6* (refer to Fig. 5 for representative hematoxylin and eosin [H&E] and MMR-IHC images). A complete or

Table
Clinicopathologic and molecular characteristics of the 84 included MMRd EC cases not related to *MLH1* promoter hypermethylation based on the underlying cause of their MMRd state

Characteristic	Total cohort (n = 84, 100%)	LS-MMRd EC (n = 31, 100%)	DS-MMRd EC (n = 32, 100%)	U-MMRd EC (n = 21, 100%)
Trial, No. (%)				
PORTEC-1	24 (29)	7 (23)	11 (34)	6 (29)
PORTEC-2	13 (16)	9 (29)	3 (9)	1 (5)
PORTEC-3	47 (56)	15 (48)	18 (56)	14 (67)
Age at random assignment (mean, range)	60 (41-82)	61 (46-82)	60 (46-74)	59 (41-73)
Age categories, No. (%)				
<60 y	45 (54)	16 (52)	17 (53)	12 (57)
≥60 y and <75 y	36 (43)	12 (39)	15 (47)	9 (43)
≥75 y	3 (4)	3 (10)	0 (0)	0 (0)
FIGO 2009 stage, No. (%)				
I	58 (69)	23 (74)	21 (66)	14 (67)
II	12 (14)	3 (10)	5 (16)	4 (19)
III	14 (17)	5 (16)	6 (19)	3 (14)
Histologic subtype and grade, No. (%)				
EEC grade 1 or 2	43 (51)	15 (48)	17 (53)	11 (52)
EEC grade 3	21 (25)	7 (23)	7 (22)	7 (33)
Non-EEC	17 (20)	7 (23)	7 (22)	3 (14)
Mixed	3 (4)	2 (7)	1 (3)	0 (0)
Myometrial invasion, No. (%)				
<50%	30 (36)	8 (26)	15 (47)	7 (33)
≥50%	54 (64)	23 (74)	17 (53)	14 (67)
Lymphovascular space invasion, No. (%)				
Absent	56 (67)	20 (65)	24 (75)	12 (57)
Present	27 (32)	10 (32)	8 (25)	9 (43)
Uncertain	1 (1)	1 (3)	0 (0)	0 (0)
MMR-IHC expression pattern, No. (%)				
MLH1/PMS2 CL	3 (4)	0 (0)	3 (9)	0 (0)
PMS2 CL	12 (14)	9 (29)	1 (3)	2 (10)
MSH2/MSH6 CL	30 (36)	5 (16)	15 (47)	10 (48)
MSH6 CL	28 (33)	16 (52)	6 (19)	6 (29)
MLH1 SL, PMS2 CL	1 (1)	0 (0)	0 (0)	1 (5)
MSH2 SL, MSH6 SL	4 (5)	0 (0)	2 (6)	2 (10)
CL or SL of >2 MMR proteins	6 (7)	1 (3)	5 (16)	0 (0)
MMR-DGF, No. (%)				
Absent	78 (93)	25 (81)	32 (100)	21 (100)
Present	6 (7)	6 (19)	0 (0)	0 (0)
MSI status, No. (%)				
MSI-H	64 (76)	24 (77)	26 (81)	14 (67)
MSS or MSI-L	17 (20)	6 (19)	5 (16)	6 (29)
Failed	3 (4)	1 (3)	1 (3)	1 (5)
POLE-EDM mutation status, No. (%)				
Wild-type	74 (88)	30 (97)	24 (75)	20 (95)
Somatic pathogenic variant	10 (12)	1 (3)	8 (25)	1 (5)

CL, complete loss; DS, double somatic; EC, endometrial carcinoma; EEC, endometrioid endometrial carcinoma; FIGO, the International Federation of Gynecology and Obstetrics; MMR-IHC, mismatch repair-immunohistochemistry; LS, Lynch syndrome; MMRd, mismatch repair-deficient; MMR-DGF, mismatch repair-deficient gland foci; MSI-L, low microsatellite instability; MSI-H, high microsatellite instability; MSS, microsatellite stable; PORTEC, Post Operative Radiation Therapy in Endometrial Carcinoma; SL, subclonal loss; U-MMRd, unexplained mismatch repair-deficient.

subclonal loss of more than 2 MMR proteins was observed in a total of 6 cases (7%) and was more prevalent among DS-MMRd EC (16%) compared with LS-MMRd EC (3%).

Survival Analyses

The median follow-up time from randomization was 8.5 years (95% CI, 6.2-9.7 years). There were no statistically significant differences in RFS among the 3 groups: 93.5% (SE, 0.04) at 5 years for LS-MMRd EC, 96.9% (SE, 0.03) for DS-MMRd EC, and 89.3% (SE, 0.07) for U-MMRd EC (*P* log-rank = .55). Similarly, for OS, no significant differences were observed among the 3 groups: 90.0% (SE, 0.06) at 5 years for LS-MMRd EC, 100% (SE, 0.00) for DS-MMRd EC, and 94.4% (SE, 0.05) for U-MMRd EC (*P* log-rank = .36) (Fig. 6). These results remained consistent for both RFS and OS when MMRd EC cases with somatic *POLE*-EDM PV were excluded (n = 10/84), as shown in Supplementary Figure S2.

Discussion

Our study identified the different underlying causes of MMRd EC not related to *MLH1*-PHM, and evaluated the differences in clinicopathologic features and prognosis between these various etiologies. Using paired tumor–normal tissue NGS, we found that 37% of cases were attributed to LS-MMRd EC, 38% were DS-MMRd EC, and the remaining 25% were U-MMRd EC. The presence of

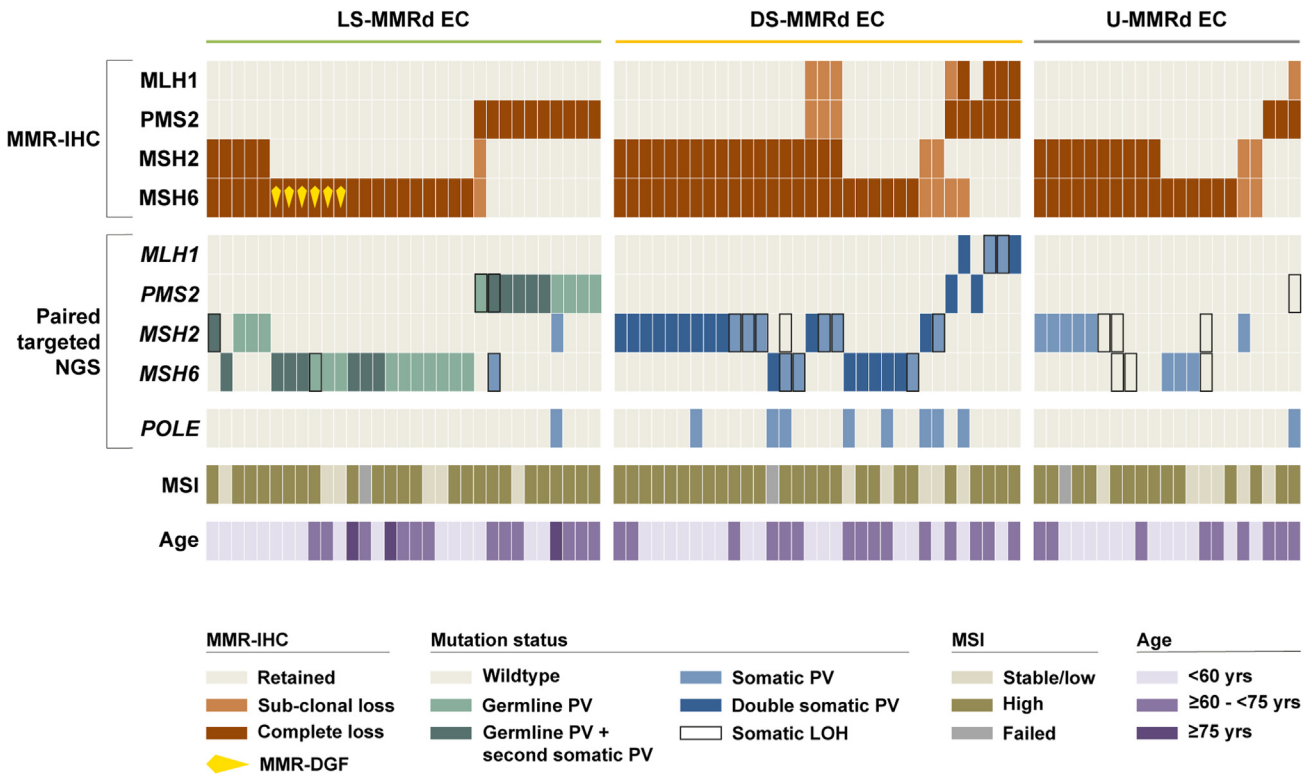

Figure 4.
Heatmap of clinicopathologic and molecular characteristics of the 84 included MMRd EC cases not related to *MLH1* promoter hypermethylation based on the underlying cause of their MMRd state. Only genetic alterations relevant for classification (as specified in the Methods) are depicted. The arrangement of cases, from left to right, follows the sequential numbering of case ID from #1 to #84. DS, double somatic; EC, endometrial carcinoma; MMR-DGF, mismatch repair-deficient gland foci; LOH, loss of heterozygosity; LS, Lynch syndrome; MMRd, mismatch repair-deficient; MSI, microsatellite instability; NGS, next-generation sequencing; PV, pathogenic variant; U-MMRd, unexplained mismatch repair-deficient.

MMR-DGF in adjacent normal endometrial glands and isolated loss of PMS2 or MSH6 were correlated with LS-MMRd EC. Conversely, the combined loss of MSH2/MSH6, loss of more than 2 MMR proteins, and somatic *POLE*-EDM PV were associated with DS-MMRd EC. We found no significant differences in PFS and OS between LS-MMRd EC, DS-MMRd EC, and U-MMRd EC. These findings carry significant implications for the diagnosis and clinical guidance of patients with MMRd EC not related to *MLH1*-PHM who are referred to clinical geneticists due to suspected LS.

When comparing our study results with previous studies, we observed similar frequencies for various causes of MMRd EC that are not related to *MLH1*-PHM. These studies also used cohorts without prior negative germline testing and adopted the same definition for DS-MMRd EC as in our study. Hampel et al[39] reported a 45% prevalence for LS-MMRd EC and 55% for DS-MMRd EC, involving a total of 22 patients. Another study with a larger cohort of 64 patients found LS-MMRd EC at 39% and DS-MMRd EC at 61%, but they excluded cases with a pathogenic *POLE* mutation (n = 6), potentially impacting the comparison.[19] Furthermore, a study involving 151 patients reported a prevalence of 30% for LS-MMRd EC, 32% for DS-MMRd EC, and 38% for U-MMRd EC.[18] It is important to note that the latter study did not include a pathology review or validation of mutation results, potentially contributing to the higher prevalence of U-MMRd EC. After excluding LS-MMRd EC, our study found a prevalence of 60% for DS-MMRd EC. When comparing our results with cohorts with prior negative germline testing, we observed similar rates of DS-MMRd EC, ranging from approximately 65% to 75%.[23,24,26] In summary, we not only validated the differentiation of MMRd EC not related to *MLH1*-PHM into LS-MMRd EC, DS-MMRd EC, and U-MMRd EC within a large EC cohort of 84 patients, but our work also underscores the efficacy of paired tumor–normal tissue NGS in finding a definitive sporadic cause, as evidenced by the consistency of our findings with prior research.

International guidelines have not yet incorporated tumor sequencing into the standard diagnostic workup for LS-associated tumors, despite accumulating evidence in support of this.[11,34] To the best our knowledge, only the Manchester International Consensus Group (for MMRd gynecologic tumors) and the joint British Society of Gastroenterology/Association of Coloproctology of Great Britain and Ireland/United Kingdom Cancer Genetics Group guidelines (for MMRd CRC) recommend tumor sequencing when no germline PV is detected in LS risk assessment.[9,53] However, these guidelines lack specific details about the optimal strategy or interpretation of tumor sequencing results. Furthermore, the National Comprehensive Cancer Network guidelines briefly mention, but do not recommend, tumor sequencing in MMRd CRC with negative germline testing. When double-somatic hits are identified, the National Comprehensive Cancer Network suggests, similar to the British Society of Gastroenterology/Association of Coloproctology of Great Britain and Ireland/United Kingdom Cancer Genetics Group guidelines, considering family history for surveillance recommendations in cases of DS-MMRd tumors, as indicated in a small footnote.[10] However, these guidelines can be ambiguous as they raise questions about the potential exemption from cancer-site surveillance recommendations for patients with DS-MMRd tumors. Partly due to these guideline limitations, the current clinical practice does not routinely include

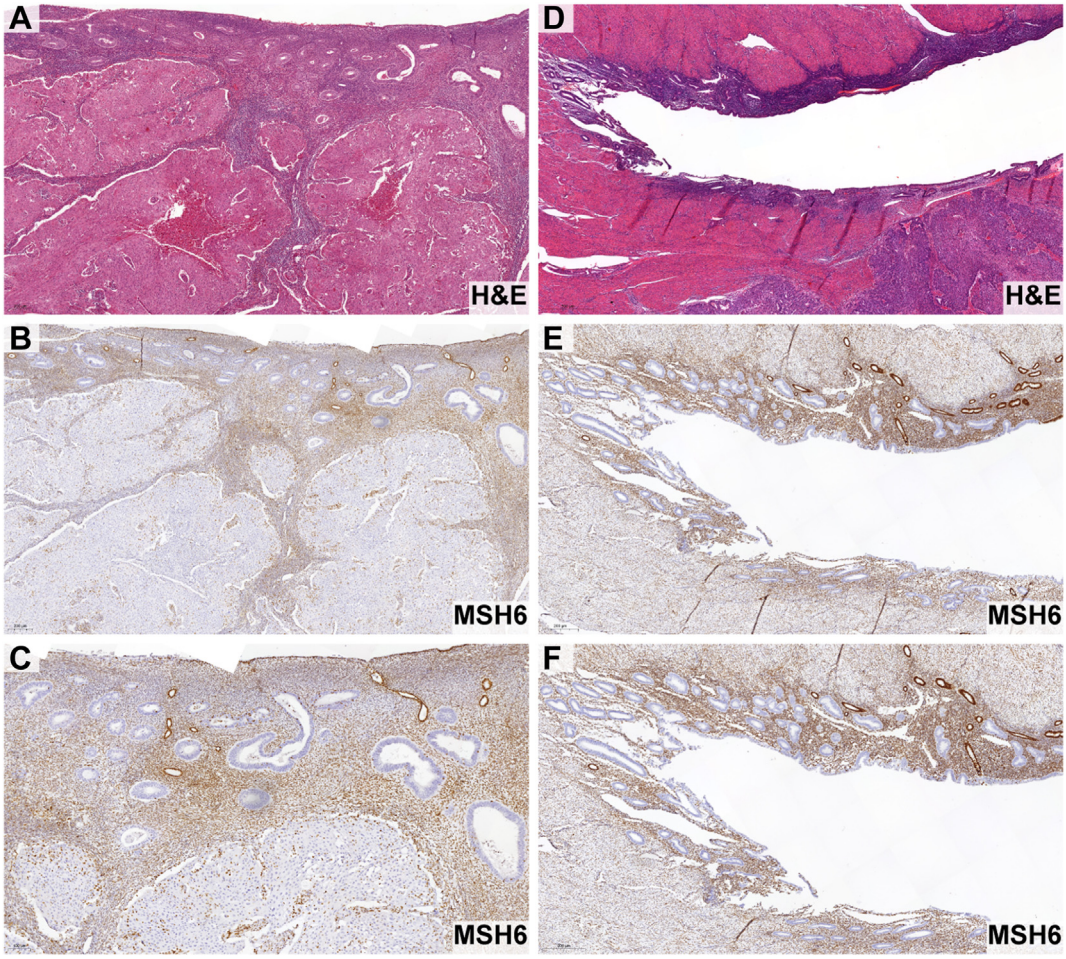

Figure 5.
Two representative LS-MMRd EC cases (#9, A-C; #8, D-F) with MMR-deficient gland foci due to germline pathogenic variants in *MSH6*. MMR-IHC shows (mutation-specific) loss of MSH6 in a cluster of glands, whereas MSH2, PMS2, and MLH1 are retained (not shown). MMR-deficient gland foci are adjacent to EC and appear morphologically indistinguishable from normal endometrial glands on H&E staining. ×4 magnification (A, B, D, E); ×7 magnification (C, F).

tumor sequencing for those with negative germline testing results.[33,37] Moreover, when tumor sequencing is performed and double-somatic hits are identified, physicians may hesitate to definitively categorize the case as sporadic. In such instances, LS surveillance may be recommended based more on physician preferences rather than an evidence-based risk approach.[7] Furthermore, the lack of national LS guidelines for the use of tumor sequencing raises additional challenges, including billing and insurance issues, often favoring germline-only testing.[33] Given the increasing evidence supporting the importance of DS-MMRd EC, international working groups must create comprehensive guidelines that incorporate tumor sequencing into germline sequencing and offer clear recommendations regarding the optimal strategy for ordering tumor sequencing and interpreting results in the context of surveillance recommendations when double-somatic hits are identified.

It was not possible to identify the underlying cause of MMR deficiency (U-MMRd) in 25% of our cohort, despite the adequate quality of the paired tumor–normal tissue NGS. In approximately half of these cases, a single-somatic hit in an MMR gene in concordance with the loss of protein expression was found, strongly suggesting that a second somatic hit may have been missed. LOH assessment of the MMR genes was not informative in 8 of 21 U-MMRd EC due to the absence of informative markers. Other explanations for the absence of a second somatic alteration may involve complex genomic rearrangements such as deletions (eg, *MSH2* exon 1-8, *PMS2* exon 9-10)[6] and inversions (eg, *MSH2* exons 1-7),[54] and deep-intronic variants not covered by our NGS approach as have been recently shown to account for up to one-third of U-MMRd cases.[28] Furthermore, the interpretation of *PMS2* variants can be challenging due to the presence of *PMS2* pseudogenes, which can lead to false-negative results, further complicated by the fragmented DNA from FFPE tissues.[55] Lastly, it is plausible that certain variants in MMR genes may contribute to disease development but are either unclassified or variants of unknown significance, as was the case in 4 of our U-MMRd EC cases. Further investigation using advanced techniques such as RNA sequencing and splicing analysis, as highlighted by previous studies, holds promise to demonstrate the pathogenicity of such variants, leading to improved variant classification and potentially increasing the diagnostic yield of paired tumor–normal NGS.[56-59]

The MMR status of our cohort was re-reviewed to exclude the possibility of false-positive cases, specifically within the U-MMRd EC subset, confirming the absence of ambiguous MMRd cases by IHC. Additionally, we conducted an MSI assay using the pentaplex panel. Notably, approximately 30% of U-MMRd EC cases had discordant MSI results (either MSS or MSI-L), a rate significantly higher than the reported 1% to 10% discordance rate between

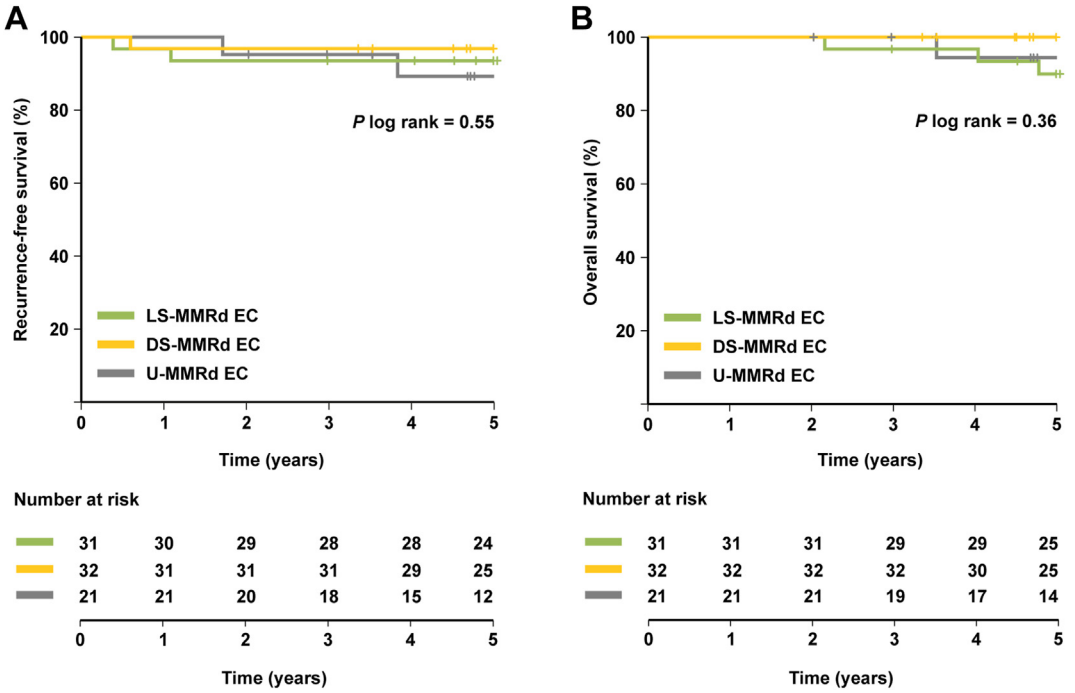

Figure 6.
Kaplan–Meier survival curves for recurrence-free survival (A) and overall survival (B) for individuals with LS-MMRd EC, DS-MMRd EC, and U-MMRd EC. The *P* value reflects the 2-sided log-rank test. DS, double somatic; EC, endometrial carcinoma; LS, Lynch syndrome; MMRd, mismatch repair-deficient; U-MMRd, unexplained mismatch repair-deficient.

MMR-IHC and MSI assay in unselected EC cohorts.[45,60] However, similar relatively high discordance rates were also observed in LS-MMRd EC and DS-MMRd EC, each showing a discordance rate of approximately 20%. This suggests a cohort-based influence rather than misclassification within U-MMRd EC cases. The lower concordance rate within this specific cohort can be partially attributed to the exclusion of cases with *MLH1*-PHM, as evidenced by a previous study. This study reported a 94% agreement between MMRd-IHC and MSI-H phenotype in unselected EC cases (n = 306/325), which decreased to 77% upon their exclusion (n = 17/22).[39] Furthermore, among the discordant MMRd and MSS/MSI-L cases within our cohort, the majority displayed MSH6 loss and subclonal MMR protein expression by IHC, consistent with prior studies.[39,61] One potential explanation is that MSI-H EC, particularly those with MSH6 loss or low tumor cellularity, may exhibit minimal microsatellite shifts, potentially evading detection by MSI-PCR tests primarily validated for MSI-H CRC.[62,63] To address this issue, it has been suggested to incorporate long mononucleotide repeat markers into a new MSI assay, which could enhance sensitivity across various non-CRC tumors, including EC.[64,65]

Certain features can help distinguish between LS-MMRd EC and DS-MMRd EC. We demonstrated that LS-MMRd EC more frequently displayed isolated loss of MSH6 or PMS2 when compared with DS-MMRd EC, consistent with previous studies.[17,22,38] Furthermore, in our cohort, DS-MMRd EC more frequently exhibited combined MSH2/MSH6 loss compared with LS-MMRd EC, in line with prior research.[18,39] The increased frequency of combined MSH2/MSH6 loss may be a more distinguishing feature of DS-MMRd EC, as indicated by a study conducted by Salvador et al,[18] where the contrast in prevalence was less notable in CRC and the combined EC/CRC cohorts when compared with EC alone. We also found that (subclonal or complete) loss of expression of more than 2 MMR proteins was more common among DS-MMRd EC. In contrast, a single study with a small sample size reported an increased prevalence of unusual MMR protein staining patterns, including subclonal loss and loss of more than 2 MMR proteins, among LS-associated tumors. However, the majority of cases (n = 11/15) were CRC, with only 1 EC case, which may limit its generalizability to EC.[66] Furthermore, a significant proportion of cases displayed MMR-DGF and were exclusively observed in LS-MMRd EC. Initially introduced as MMR-deficient crypt foci in LS-associated CRC, studies have found that MMR-deficient gland formation is unique to LS-MMRd EC, supporting its potential as a diagnostic marker.[49,67-69] In contrast, a lower frequency of MMR-DGF has been reported in DS-MMRd EC, although still less frequent than in LS-MMRd EC (9% vs 67%, respectively).[70] In summary, our study confirms that isolated loss of MSH6 or PMS2 is a frequent event in LS-MMRd EC, and suggests that combined MSH2/MSH6 loss and loss of expression of more than 2 MMR proteins is commonly associated with DS-MMRd EC. Furthermore, our findings underscore the potential utility of MMR-DGF as a supporting factor for identifying LS-MMRd EC.

Apart from MMR-IHC results, the *POLE*-EDM mutation status can also help in distinguishing between a hereditary and sporadic cause of MMR deficiency in EC. Somatic *POLE*-EDM PV were observed in 12% of our cohort, and their prevalence was higher in DS-MMRd EC than in LS-MMRd EC. A study by Haraldsdottir et al,[21] which included both MMRd CRC and EC, reported a prevalence of 16% for somatic *POLE*-EDM PV, which is in line with our findings. Billingsley et al[30] reported a prevalence of 18% in MMRd EC but included *POLE* variants outside of the exonuclease domain of *POLE*, which would not qualify as *POLE*-mutant EC. Both these prevalence rates were reported in cases not related to *MLH1*-PHM.[21,30] The molecular class assignment of EC is incorporated in the diagnostic algorithm described in the WHO 2020 classification of tumors.[71] According to this algorithm, cases with somatic pathogenic *POLE*-EDM hot spot variants and loss of MMR

function are classified as *POLE*-mutant EC. These tumors are classified based on their genomic and prognostic similarities to those with impaired *POLE* proofreading rather than loss of MMR function.[2,72] A somatic *POLE*-EDM PV found in the context of LS is extremely rare, and in this scenario, it may be argued that the tumor has a sporadic origin without a causal relationship to the germline MMR variant.[21,29,73] In our study, we identified only 1 LS-MMRd EC with a co-occurring germline PV in *PMS2* and a somatic *POLE*-EDM PV. To the best of our knowledge, there has been only 1 reported case where both a germline PV in an MMR gene and a somatic *POLE*-EDM PV co-existed, and this case was observed in glioma.[74] Based on the high frequency of *POLE*-EDM PV observed in DS-MMRd EC, our findings support the previously suggested notion that somatic *POLE*-EDM PV, in the absence of a germline PV, can be considered evidence in favor of a sporadic origin.[29,30] Validation of this observation is warranted, as the introduction of somatic *POLE* testing as an indicator of sporadic origin in novel guidelines for LS could be considered.

No significant differences in other clinicopathologic characteristics including age, histologic subtype and grade, or prognosis were observed between LS-associated and DS-MMRd EC. It is worth noting that DS-MMRd EC, similar to LS-associated MMR-deficient EC, exhibited an early age of onset, consistent with mean ages reported in previous studies, ranging from 52 to 58 years.[19,22,24,26] These findings highlight the limitations of existing guidelines that use age as the primary criterion to assess the likelihood of genetic predisposition to LS in individuals with MMRd tumors who have tested negative for germline PV.[53] Relying solely on age could lead to unnecessary surveillance for both cancer-affected individuals and their families, further emphasizing the importance of integrating tumor sequencing with germline sequencing into LS diagnostic protocols to detect double-somatic hits. Furthermore, we observed a favorable prognosis in RFS and OS across LS-MMRd EC, DS-MMRd EC, and U-MMRd EC. In another single study with a median follow-up of 25 months, both LS-MMRd EC and DS-MMRd EC demonstrated a favorable PFS that was comparable, with no significant difference between them.[19] In summary, our study confirmed an overall good prognosis for patients with MMRd EC not related to *MLH1*-PHM, with no difference observed among cases with hereditary, sporadic, or unexplained MMRd origins.

Our study has several limitations. First, it is important to acknowledge that the eligibility criteria in the PORTEC trials potentially introduced selection bias. Specifically, patients with stage IA grade 1 and less than half myometrial invasion were not represented in our combined PORTEC-1, -2, and -3 clinical trial cohort. Additionally, the PORTEC-2 trial used an age cutoff as part of its inclusion criteria; however, only 16% of our cohort originated from PORTEC-2.[75] Furthermore, the PORTEC trials excluded those with a prior history of cancer. Given that individuals with a germline PV in *MLH1* are associated with an increased risk of early-onset CRC, this exclusion may have led to their absence in our study.[76] Second, the limited quality and availability of DNA samples, particularly due to the use of relatively old tumor material, resulted in a 14% failure rate in NGS analysis. This limitation also led to incomplete MS-MLPA analysis, potentially causing us to miss certain epigenetic alterations or *EPCAM* deletions in some unexplained cases. However, given the reported low prevalence of promoter hypermethylation in MMR genes other than *MLH1*, with only 1 case reported in EC, and the low prevalence of *EPCAM* deletions, the impact of these missed alterations on our findings is expected to be minimal.[22] Third, the inability to definitively determine whether double-somatic PV in MMRd EC occur in cis (same allele) or trans (different alleles) is a limitation. Nonetheless, when 2 PV are identified in different amplicons, it is reasonable to assume that they are located on different alleles. Last, the absence of family history and DNA from different normal tissues (eg, blood, and saliva) prevented us from excluding the possibility of mosaicism in patients with double-somatic and single-somatic PV. However, based on the rare prevalence of de novo variants in DNA MMR genes reported in EC, we do not anticipate a significant impact.[77,78]

To our knowledge, our study is the first to comprehensively investigate the underlying cause of MMRd among a large cohort of patients with MMRd EC not related to *MLH1*-PHM, whereas simultaneously determining the correlations with clinicopathologic characteristics and prognosis. We provide evidence that a significant proportion of these tumors have a sporadic origin due to double-somatic hits using paired tumor–normal tissue NGS. This underscores the urgency of developing comprehensive international guidelines on the integration of tumor sequencing alongside germline sequencing into the diagnostic workup of LS. The assessment of MMR protein expression through IHC, associated affected MMR genes, and the *POLE* mutational status were informative in distinguishing between LS-MMRd EC and DS-MMRd EC. Although the distinction between LS-MMRd EC, DS-MMRd EC, and U-MMRd EC did not have prognostic significance, it is informative for clinical management. When a DS-MMRd EC case is confirmed, genetic cascade testing is unnecessary, and intensive LS surveillance can be avoided, alleviating significant distress on cancer-affected individuals and their at-risk relatives.

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Author Contributions

M.K., C.C.B.P., T.B., V.T.H.B.M.S., and C.L.C. conceptualized the study. M.K., C.C.B.P., and T.B. carried out experiments. M.K., T.V.W., C.T., and N.H. analyzed the data. All authors were involved in writing the paper and had final approval of the submitted and published version.

Data Availability

The data underlying this article are available from the corresponding author on reasonable request.

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Declaration of Competing Interest

The authors have declared no conflict of interest.

Ethics Approval and Consent to Participate

Translational research was performed on tissue samples from 3 randomized clinical trials that were approved and granted by the Scientific Committee of the Dutch Cancer Society (KWF

Kankerbestrijding) (grant numbers CKTO 90−01, CKTO 2001−04, and UL2006-4168/CKTO 2006−04) and by the Ethics Committees of LUMC and of the participating centers. Specific Ethics approval was obtained for variant analysis on normal tissue among those suspected of LS (METC-LDD C16. 027).

Supplementary Material

The online version contains supplementary material available at https://doi.org/10.1016/j.modpat.2024.100423.

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