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Original Article

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MLH1 promoter hypermethylation predicts poorer prognosis in mismatch repair deficiency endometrial carcinomas

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ABSTRACT

Objective: The antitumor effects of anti-PD-1 antibody against mismatch repair deficiency (MMR-D)-associated cancers have been reported. MMR-D is found in approximately 20%–30% of endometrial carcinomas (ECs) and frequently occurs due to *MLH1* promoter hypermethylation (*MLH1*-PHM). ECs with *MLH1*-PHM are classified according to the molecular screening of Lynch syndrome (LS), but few detailed reports are available. The purpose of this study was to clarify the clinical features of EC with *MLH1*-PHM.
Methods: Immunohistochemistry of MMR proteins (MLH1, MSH2, MSH6, and PMS2) was performed on specimens from 527 ECs treated at our university hospital from 2003 to 2018. *MLH1* methylation analysis was added to cases with MLH1/PMS2 loss. ECs were classified as follows: cases that retained MMR proteins as "MMR-proficient;" cases with MLH1/PMS2 loss and *MLH1*-PHM as "met-EC;" and cases with other MMR protein loss and MLH1/PMS2 loss without *MLH1*-PHM as "suspected-LS." The clinical features, including long-term prognosis, of each group, were analyzed.
Results: Accordingly, 419 (79.5%), 65 (12.3%), and 43 (8.2%) cases were categorized as "MMR-proficient," "suspected-LS," and "met-EC," respectively. Significantly, "met-EC" had a lower proportion of grade 1 tumors (37.5%) and a higher proportion of stage III/IV tumors (37.2%) than the other groups. The overall and progression-free survival of "met-EC" were significantly worse than those of "suspected-LS" in all cases.
Conclusion: In ECs with MMR-D, "met-ECs" were a subgroup with a poorer prognosis than "suspected-LS." "Met-ECs" would be the main target for anti-PD-1 antibody treatment, and its clinical susceptibility should be verified individually.

Keywords: Endometrial Neoplasms; DNA Mismatch Repair; Methylation; Prognosis

INTRODUCTION

Anti-PD-1 antibody, an immune checkpoint inhibitor, has been reported to show high antitumor effects against various cancers associated with mismatch repair deficiency (MMR-D) [1]. MMR-D has drawn attention as a carcinogenic mechanism. The proteins MLH1, MSH2, MSH6, and PMS2 play important roles in the mechanism of MMR. MMR-D

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Author Contributions
Conceptualization: K.E., S.N., S.T., N.A.; Data curation: K.E., S.N., S.T., N.A., T.K.; Formal analysis: K.E., S.N., S.T.; Funding acquisition: S.N., S.T., T.Y.; Investigation: K.E., S.N., S.T., N.A., T.K., M.K.; Methodology: K.E., S.N., N.A., T.K.; Project administration: K.E., S.N., T.Y.; Resources: K.E., S.N., S.T., N.A., T.K., M.K.; Software: K.E.; Supervision: T.Y.; Validation: K.E., S.N., M.K., T.Y.; Visualization: K.E., S.N.; Writing - original draft: K.E., S.N.; Writing - review & editing: K.E., S.N., S.T., N.A., T.K., M.K., T.Y.

causes the accumulation of gene mutations in somatic cells, leading to carcinogenesis. MMR-D is detected by immunohistochemistry (IHC) or microsatellite instability tests, and has been identified in 6%–14% of colorectal cancers (CRC) [1-3] and 17%–40% of endometrial carcinomas (ECs) [1,4-9].

ECs with MMR-D are classified into 3 groups: Lynch syndrome (LS), Lynch-like cases, and cases with *MLH1* promoter hypermethylation (*MLH1*-PHM). LS is an autosomal dominant hereditary disorder caused by MMR-D due to the addition of a somatic mutation in the contralateral allele to a pathogenic germline mutation in one of the MMR genes. In Lynch-like cases, pathogenic MMR gene mutations are not detected in the germline DNA despite presenting MMR-D. In the majority of Lynch-like cases, bi-allelic somatic mutations are found in MMR genes [2,10]. In cases with *MLH1*-PHM, MMR-D is caused by the silencing of *MLH1*. *MLH1*-PHM is found in 61%–80% of ECs with MMR-D [4,8,9,11,12] and is one of the major carcinogenic mechanisms underlying ECs.

We have devised an efficient LS screening strategy that incorporates the original triage [13]. By performing universal molecular screening and genetic testing, we detailed the clinical features of the LS and Lynch-like groups in Japanese ECs [14]. We also found that isolated loss of PMS2 is frequently caused by *MLH1*-PHM [15]. ECs with *MLH1*-PHM have been treated as sporadic cancer with MMR-proficient ECs. However, ECs with *MLH1*-PHM exhibit MMR-D and can be a target for anti-PD-1 antibodies, and therefore can be clinically distinguished from MMR-proficient ECs. Molecular and pathological characteristics of ECs with *MLH1*-PHM were analyzed in some reports [7-9], but few describe their clinical picture in detail. The purpose of this study was to explore and describe the clinical features of ECs with *MLH1*-PHM.

MATERIALS AND METHODS

1. Study population and procedures

Of the 545 patients diagnosed with ECs at the Akita University Hospital from January 2003 to December 2018, 527 patients with evaluable tumor tissue were retrospectively analyzed. Seventeen cases were excluded from this study because of insufficient tumor tissue volume for MMR-IHC, and one case was excluded because of insufficient tumor tissue volume for *MLH1* methylation analysis. All patients were Asians living in Japan. Patients' clinical data were collected from medical records and clinical inquiries. The family history of LS-associated cancers was collected from first- and second-degree relatives. This study population included 180 newly diagnosed patients with ECs from January 2014 to December 2018, in addition to the 348 participants in our previous study [13-15]. Information on participants in previous studies was revised by additional surveys. MMR-IHC was performed on the tumors of all ECs to assess MMR protein expression. *MLH1* methylation analysis was performed on MLH1 and/or PMS2 deficient tumors. (**Fig. 1A**) All study participants provided written informed consent. The Institutional Review Board of Akita University approved the study design (IRB No.1273).

2. MMR-IHC

Following standard procedures, MMR-IHC was performed to assess the expression of MMR proteins (MLH1, MSH2, MSH6, and PMS2) in tumors of all EC patients. An appropriate paraffin-embedded tissue was cut to 4 μm-thickness. The tissue sections were deparaffinized with xylene and rehydrated in graded alcohol. Antigen retrieval was performed in 10 mmol/L

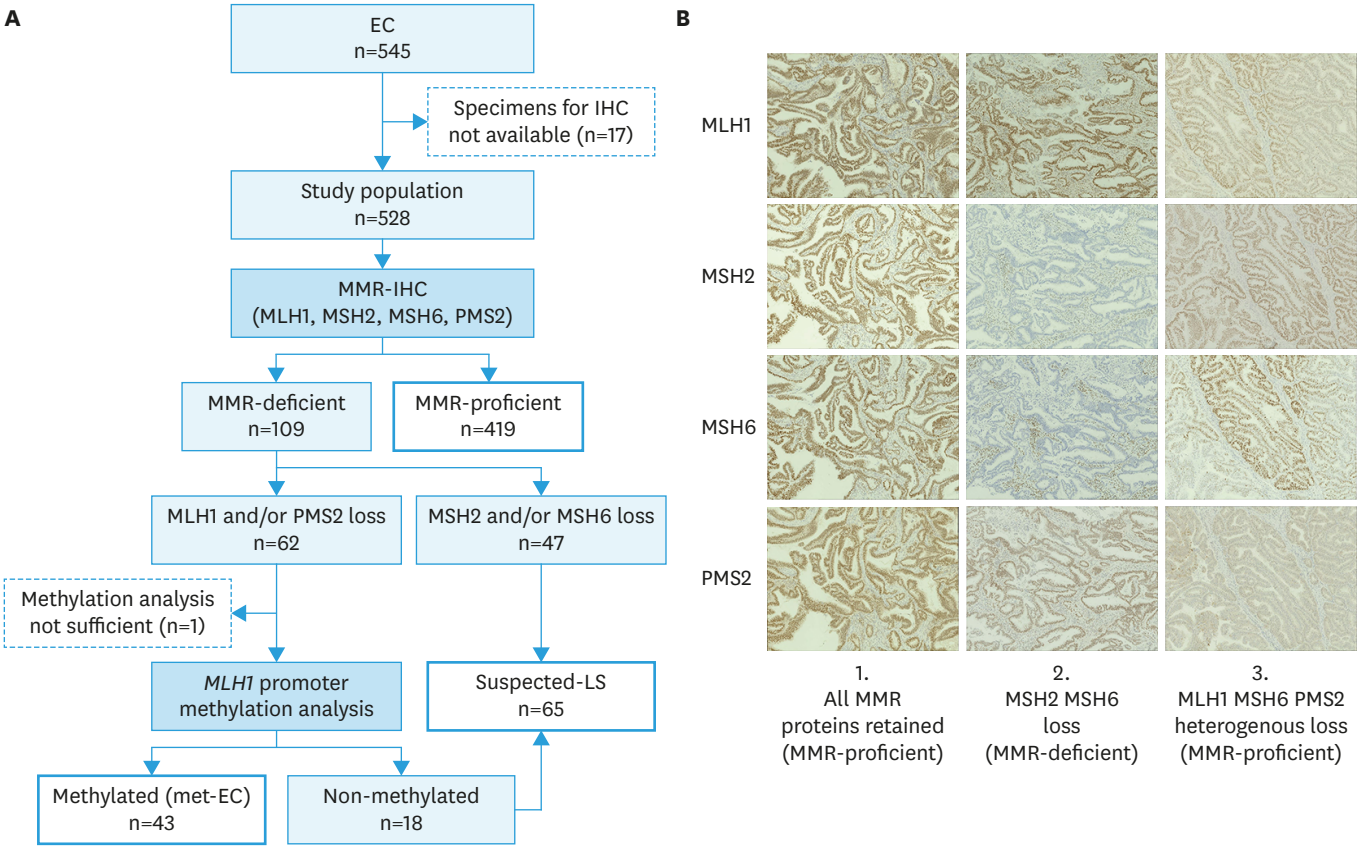

Fig. 1. Summary of this study. (A) Flowchart of classification. (B) Representative IHC photos of MMR expression.
EC, endometrial carcinoma; IHC, immunohistochemistry; LS, Lynch syndrome; met-EC, endometrial carcinoma with *MLH1* promoter hypermethylation; MMR, mismatch repair.

Tris-EDTA buffer (pH 9.0) in a microwave oven for 20 minutes. The sections were cooled to room temperature. The primary antibody was added overnight at 4°C. The following primary antibodies were used: MLH1 (clone ES05; dilution 1:50; Dako, Glostrup, Denmark), MSH2 (clone FE11; dilution 1:50; Dako), MSH6 (clone EP49; dilution 1:50; Dako), and PMS2 (clone EP51; dilution 1:40; Dako). The antigen-antibody reaction was visualized using the Envision kit (Dako). The slides were counterstained with hematoxylin. Adjacent normal endometrium and lymphocytes in the section were used as internal positive controls. Representative IHC photos of MMR expression were shown in **Fig. 1B**. According to the standard screening methods for LS, cases with a complete absence of nuclear staining in whole sections were judged as "loss of MMR protein expression."

3. MLH1 promoter methylation analysis

We previously reported that isolated loss of PMS2 expression observed by MMR-IHC was often caused by *MLH1*-PHM [15]. Therefore, *MLH1* promoter methylation analysis was performed on MLH1 and/or PMS2 deficient tumors. The tumor DNA was extracted from mapped formalin-fixed, paraffin-embedded tissue sections to provide tumor samples for the assays. The SALSA MS-MLPA mismatch repair genes kit (ME011; MRC-Holland, Amsterdam, The Netherlands), which contains 5 probes recognizing *MLH1*, was used to detect aberrant CpG island methylation in MMR gene promoters. The MS-MLPA assay was performed according to the manufacturer's instructions. We focused on the promoter C region (probe 3), which provides the best correlation with MLH1 expression [16]. Based on a previous study

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associated with gene silencing, the threshold for distinguishing between hypermethylated and non-methylated genes was set at 15% [17].

4. Classification

Cases that retained MMR protein expression in IHC were classified as "MMR-proficient." Cases with loss of MLH1 and/or PMS2 and confirmed *MLH1* hypermethylation were classified as "met-EC." Cases with at least one MMR protein loss not caused by *MLH1*-PHM were classified as "suspected-LS."

5. Statistical analysis

The clinical features of "MMR-proficient," "suspected-LS," and "met-EC" were statistically compared using the χ^2 test or Fisher's exact test (2-sided). Overall survival (OS) and progression-free survival (PFS) were analyzed using the Kaplan-Meier method, and the results were compared using the log-rank test. Multivariate analyses for prognostic factors were performed using Cox proportional hazard model. Statistical significance was defined as $p<0.05$. All data were analyzed using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA).

RESULTS

MMR-IHC was performed on tumor tissue obtained from 528 patients with ECs having evaluable specimens. The clinical features of the EC cases examined in this study are shown in **Table 1**. The 419 cases (419/528, 79.4%) that retained all MMR protein expressions were classified as "MMR-proficient." Besides, 109 cases (109/528, 20.6%) showed the loss of at least one MMR protein. The MMR protein loss pattern is shown in **Table 1**. Methylation analysis of the *MLH1* promoter region was performed on 61 patients with MLH1 and/or PMS2 loss, and 43 cases with *MLH1*-PHM were classified as "met-EC" (one case was excluded because of insufficient data). "Met-EC" accounted for 8.2% (43/527) of ECs examined, 39.8% (43/108) of cases with MMR-D, and 70.5% (43/61) of cases with loss of MLH1 and/or PMS2. A total of 65 cases, including 18 cases of MLH1 and/or PMS2 loss without *MLH1*-PHM, 36 cases of MSH2 and/or MSH6 loss, and 11 cases showing other MMR protein loss patterns, were classified as "suspected-LS." Other patterns of MMR protein loss are summarized in **Table 1**.

The clinical features of "MMR-proficient," "suspected-LS," and "met-EC" are shown in **Table 2**. The proportion of patients under 50 years of age at EC onset was significantly higher in "suspected-LS" cases (27.7%, 18/65) than in "MMR-proficient" cases (16.2%, 68/419). Among the "MMR-proficient" cases, the proportion of patients with body mass index >30 was significantly higher than that of "suspected-LS" cases (p=0.045). Endometrioid type carcinoma accounted for more than 80% in each group. The percentage of pathological grade in the endometrioid type is shown in **Fig. 2A**. In "met-EC," the proportion of G1 was 37.5%, significantly lower than that of the other groups ("MMR-proficient" 60.3%, p=0.005; "suspected-LS" 58.9%, p=0.038), and the proportion of G2 was 42.5%, which was significantly higher than that of the "MMR-proficient" group (26.8%, p=0.039). The proportion of stage III/IV tumors in "met-EC" was 37.2%, significantly higher than that in the other groups ("MMR-proficient" 22.7%, p=0.034; "suspected-LS" 15.4%, p=0.009) (**Fig. 2B**). The personal incidence of CRC was significantly higher in the "suspected-LS" group (13.8%, 9/65) than in the "MMR-proficient" (2.4%, 10/419) and "met-EC" (2.3%, 1/43) groups (**Fig. 2C**). The prevalence of family history of CRC was significantly higher in the "suspected-LS" group (36.9%, 24/65) than in the "MMR-proficient" (10.5%, 44/419) and "met-EC" groups (16.3%, 7/43) (**Fig. 2D**).

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Table 1. Clinicopathologic data of the study cohort (n=528)

Variables	Value
Age at diagnosis of EC	58.8±11.2
BMI (kg/cm^2)	23.6 (14.7–43.6)
Histological type	
Endometrioid	436
G1	253
G2	126
G3	56
Unclassifiable	1
Mucinous	1
Serous	18
Clear cell carcinoma	18
Mix	16
Carcinosarcoma	27
Others	12
FIGO stage	
I	367
II	39
III	67
IV	55
CA125 (U/mL)	15.3 (1.5–9,419.5)
CA19-9 (U/mL)	15.1 (0.8–7,861.3)
Immunohistochemistry of MMR proteins	
MMR-proficient	419
MMR-deficient	109
MMR loss patterns	
MLH1/PMS2	43
PMS2	19
MSH2/MSH6	21
MSH6	15
Others	11
MLH1	1
MSH2	2
MLH1/MSH6	3
MLH1/MSH6/PMS2	5
MLH1 promoter methylation testing (n=62)	
Methylated	43
Non-methylated	18
Methylation analysis not available	1

Values are presented as mean±standard deviation or median (range).
BMI, body mass index; CA, cancer antigen; EC, endometrial carcinoma; FIGO, International Federation of Gynecology and Obstetrics; MMR, mismatch repair.

OS and PFS are shown in **Figs. 3** and **4**. The median follow-up period for the cohort was 70 months (range, 1–207 months), with a cumulative 5-year survival rate of 84.5%. The total number of deaths during the follow-up period was 94, and the number of deaths from EC was 71. The cumulative 5-year survival rates for each group were 83.5% for "MMR-proficient," 88.5% for "MMR-deficient," 94.6% for "suspected-LS," and 79.2% for the "met-EC" group. The OS and PFS of MMR-D cases were significantly better than those of the "MMR-proficient" group (p=0.021 and p=0.026, respectively, as shown in **Fig. 3A and B**). The OS and PFS of the "met-EC" group were significantly worse than those of the "suspected-LS" group (p=0.018 and p=0.003, respectively, as shown in **Fig. 3C and D**). Both OS and PFS of non-endometrioid type were significantly worse than those of endometrioid type (**Fig. 4A and B**). In this study, prognostic differences were not proven between cases with MMR-proficient and cases with MMR-D (**Fig. 4C and D**). Dividing MMR-D cases into "suspected-LS" and "met-EC" based on the result of *MLH1* methylation analysis, "met-EC" showed worse OS and PFS than the other 2

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Table 2. Clinicopathologic features of MMR-proficient, suspected-LS, and met-EC

Variables	MMR-proficient (n=419)	Suspected-LS (n=65)	Met-EC (n=43)	p-value		
				MMR-proficient vs. suspected-LS	MMR-proficient vs. met-EC	Suspected-LS vs. met-EC
Mean age at diagnosis of EC	59.4±11.3	55.1±10.2	58.6±10.0			
<50 years at diagnosis of EC	68 (16.2)	18 (27.7)	8 (18.6)	**0.024**	0.689	0.280
Median BMI (kg/cm²)	23.7 (14.7–43.6)	22.8 (18.1–35.5)	23.4 (16.2–39.7)			
BMI >30	65 (15.5)	4 (6.2)	3 (7.0)	**0.045**	0.132	1.000*
Histology				0.330	0.052	0.356*
Endometrioid	340 (81.1)	56 (86.2)	40 (93.0)			
Non endometrioid	79 (18.9)	9 (13.8)	3 (7.0)			
Grade in endometrioid type					Shown in **Fig. 2A**	
Low grade (type1) G1	205 (60.3)	33 (58.9)	15 (37.5)			
G2	91 (26.8)	18 (32.1)	17 (42.5)			
High grade (type2) G3	43 (12.6)	5 (8.9)	8 (20.0)			
Unclassifiable	1 (0.3)	0 (0.0)	0 (0.0)			
Stage					Shown in **Fig. 2B**	
I/II	324 (77.3)	55 (84.6)	27 (62.8)			
III/IV	95 (22.7)	10 (15.4)	16 (37.2)			
Personal medical history						
Hypertension	160 (38.2)	20 (30.8)	17 (39.5)	0.250	0.862	0.347
Diabetes	88 (21.0)	8 (12.3)	10 (23.3)	0.102	0.731	0.135
Hyperlipidemia	97 (23.2)	14 (21.5)	8 (18.6)	0.774	0.498	0.711
LSAC	23 (5.5)	15 (23.1)	4 (9.3)	**<0.001**	0.302*	0.066
Colorectal carcinoma	10 (2.4)	9 (13.8)	1 (2.3)		Shown in **Fig. 2C**	
Gastric carcinoma	9 (2.1)	5 (7.7)	2 (4.7)	**0.028***	0.273*	0.700*
Family history						
LSAC	170 (40.6)	39 (60.0)	20 (43.5)	**0.003**	0.376	0.209
Colorectal carcinoma	44 (10.5)	24 (36.9)	7 (16.3)		Shown in **Fig. 2D**	
Gastric carcinoma	101 (24.1)	20 (30.8)	14 (32.6)	0.248	0.188	0.781
Tumor marker						
CA125	15.2 (1.5–6,478.2)	17.5 (3.5–841.8)	15.4 (3.2–9,419.5)			
Elevated CA125 (>35 U/mL)	106 (25.7)	18 (28.6)	16 (37.2)	0.681	0.092	0.297
CA19-9	14.7 (0.8–7,861.3)	20.3 (0.8–1,494.0)	15.8 (0.8–4,095.0)			
Elevated CA19-9 (>37 U/mL)	93 (22.5)	19 (30.2)	12 (27.9)	0.211	0.395	0.882
Primary treatment				0.738	0.397	0.214
Operation	411 (98.1)	65 (100.0)	41 (95.4)			
Chemotherapy	4 (1.0)	0 (0.0)	1 (2.3)			
Radiation therapy	2 (0.5)	0 (0.0)	1 (2.3)			
MPA	2 (0.5)	0 (0.0)	0 (0.0)			
In operation cases	(n=411)	(n=65)	(n=41)			
Lymph node dissection				0.239	0.378	0.714
Non	94 (22.9)	9 (13.8)	6 (14.6)			
PLN	127 (30.9)	24 (36.9)	12 (29.3)			
PLN and PAN	190 (46.2)	32 (49.2)	23 (56.1)			
Neoadjuvant chemotherapy				0.450*	0.667*	0.626*
None	399 (97.1)	64 (98.5)	40 (97.6)			
Done	12 (2.9)	1 (1.5)	1 (2.4)			
Adjuvant therapy				0.482	0.216	0.137
None	202 (49.1)	35 (53.8)	17 (41.5)			
Chemotherapy	201 (48.9)	28 (43.1)	22 (53.7)			
Radiation therapy	8 (1.9)	2 (3.1)	2 (4.9)			

Values are presented as mean±standard deviation, number (%), or median (range). Bold-faced p-values are statistically significant.
BMI, body mass index; EC, endometrial carcinoma; LS, Lynch syndrome; LSAC, lynch syndrome-associated cancer; met-EC, endometrial carcinoma with *MLH1* promoter hypermethylation; MMR, mismatch repair; MPA, medroxyprogesterone acetate; PAN, para-aortic lymph node; PLN, pelvic lymph node.
When there is no mark, the χ^2 test was used. * used Fisher's exact test (2-sided).

groups (**Fig. 4E and F**). The cumulative 5-year survival rates of endometrioid type cases were 95.7% for "suspected-LS" and 76.6% for "met-EC."

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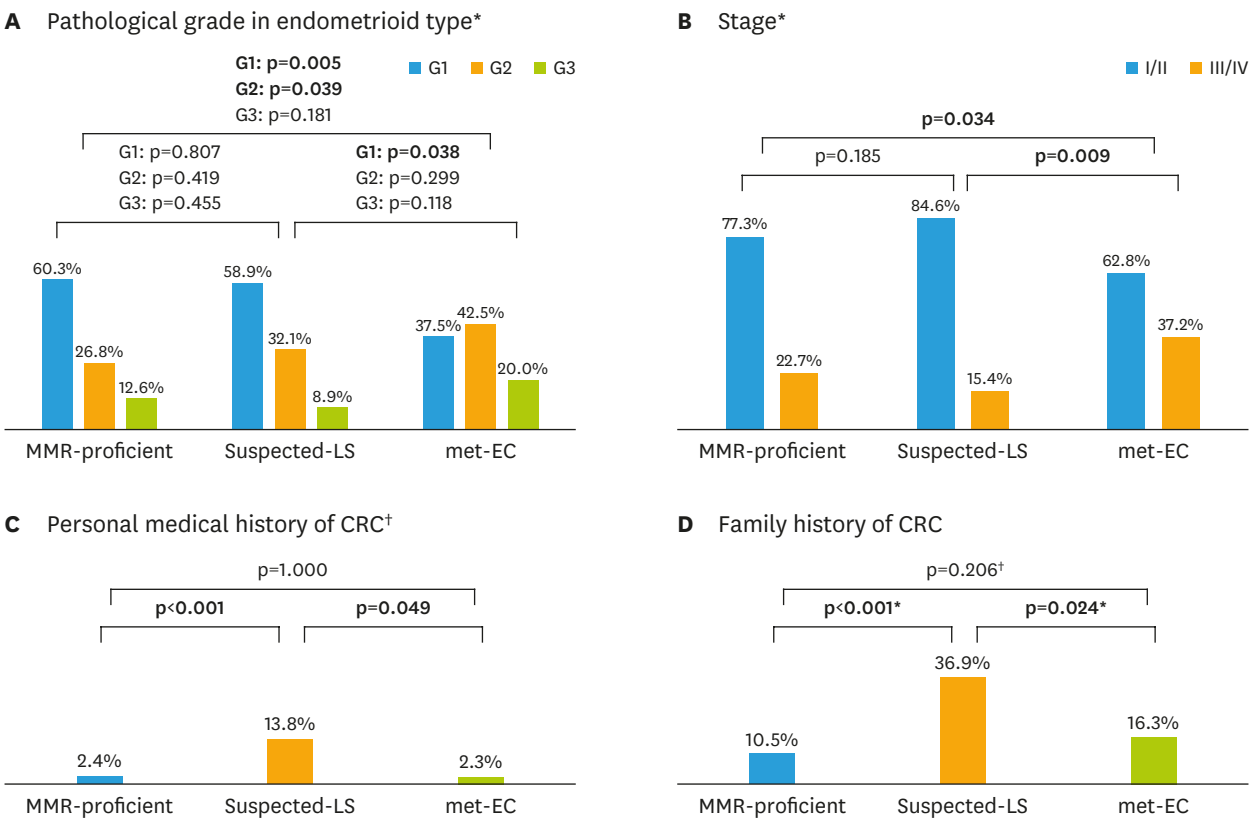

Fig. 2. Comparison among the MMR-proficient, suspected-LS, and met-EC groups. (A) Pathological grade in endometrioid type. (B) Stage. (C) Personal medical history of CRC. (D) Family history of CRC.
CRC, colorectal cancer; LS, Lynch syndrome; met-EC, endometrial carcinoma with *MLH1* promoter hypermethylation; MMR, mismatch repair.
*Chi-squared test; †Fisher's exact test (2-sided).

Performing multivariate analysis, *MLH1*-PHM was not proven to be an independent poor prognostic factor (**Table S1**). The OS and PFS of both low grade (endometrioid G1 and G2) or high grade (endometrioid G3) cases were analyzed (**Fig. S1**). In low grade cases, the PFS of "met-EC" was significantly worse than that of "suspected-LS." High grade cases showed a graphically similar trend (significant difference were not proven).

DISCUSSION

In the LS screening process, ECs are molecularly classified as "MMR-proficient," "suspected-LS," and "met-EC." This study focused on "met-EC" cases and analyzed their clinical features, including long-term prognosis. "met-EC" cases showed poorer prognosis compared with "suspected-LS" cases.

MSI is highly concordant with MMR-IHC in the judgment of MMR-D, but occasionally overlooks MSH6 or PMS2 mutations [18]. The advantages of using MMR-IHC to evaluate MMR-D in EC include (1) high sensitivity as a LS screening method, (2) subject selection for *MLH1* methylation analysis, and (3) prediction of deficient MMR genes. We believe that *MLH1* methylation analysis based on the MMR-IHC judgment will contribute to (1) improvement of positive predictive value in LS screening, (2) identification of met-EC group with a poor prognosis, and (3) verification of the target tumors for PD-1 antibody therapy.

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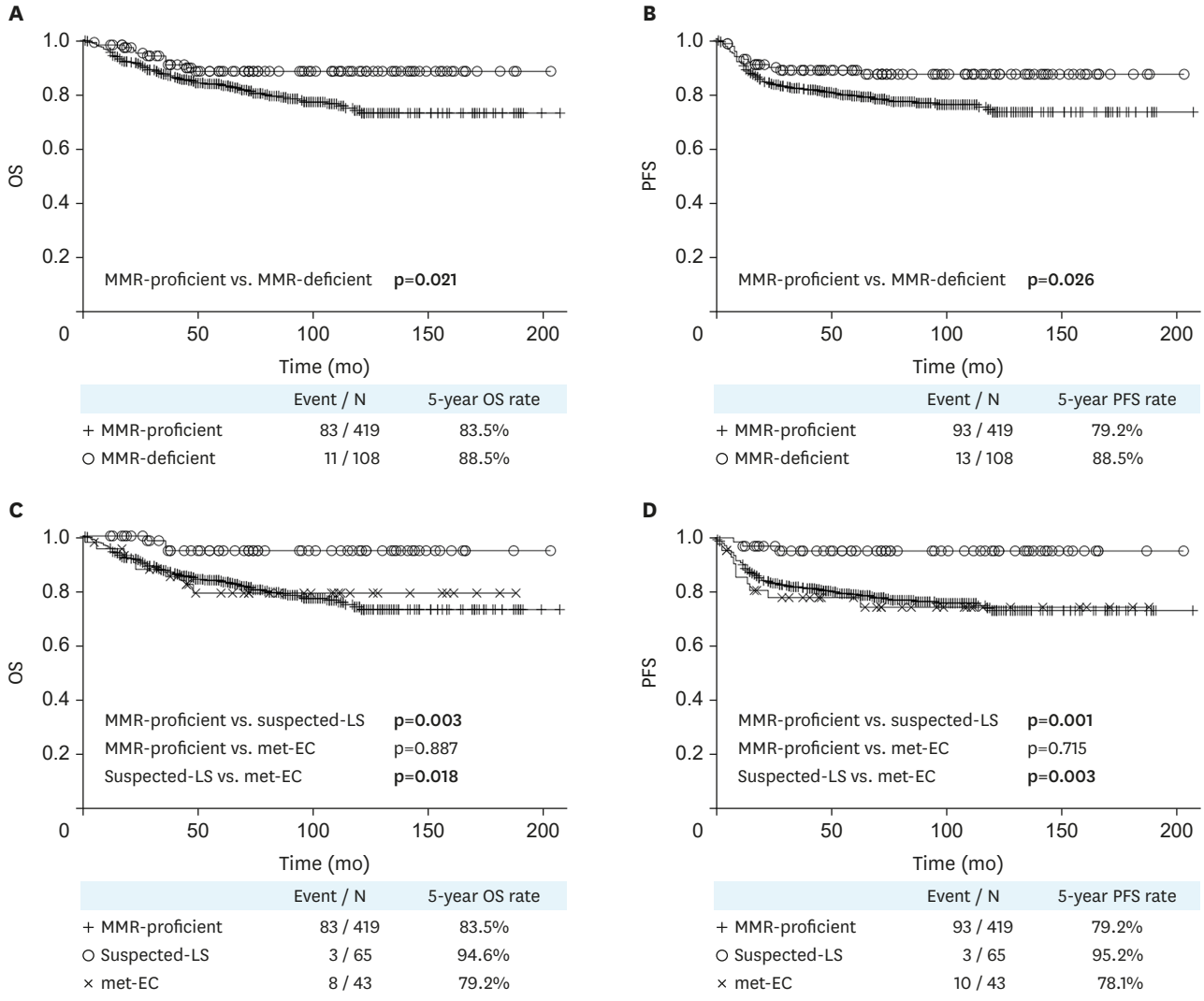

Fig. 3. Survival analysis. (A, B) OS and PFS according to MMR-status. (C, D) OS and PFS.
LS, Lynch syndrome; met-EC, endometrial carcinomas with *MLH1* promoter hypermethylation; MMR, mismatch repair OS, overall survival; PFS, progression-free survival.

The proportion of "met-EC" cases are reported to be 13%–27% of ECs [4,8,9,11], 61%–80% of ECs with MMR-D [4,8,9,11,12], and 74%–91% of MLH1 deficient cases [5,9,19]. In this study, the proportion of "met-EC" cases were 8.2% of the total ECs examined, 39.8% of the MMR-D cases, and 68.3% of cases with loss of MLH1 and/or PMS2, lower than previously reported. Although the proportion of MMR-D in this study was similar to that in other reports [4-7,9], the number of cases judged to show MLH1 and/or PMS2 loss was smaller than that in other reports [5,20]. This difference might be due to the rigor of IHC analysis. Most of specimens in this study were prepared from the excised uteruses, and the cases with heterogeneous and/or focal loss of intratumoral MMR protein expression were not judged to be MMR-deficient. Some reports have described regional differences in MMR protein deficiency distribution [9,14].

Patients with LS have a more prevalent family history of various LS-associated cancers, including CRC [21,22]. Patients with Lynch-like cases have a higher prevalence in their family history of some LS-associated cancers than sporadic EC patients [14,23]. In other words, "suspected-LS" cases tend to have a hereditary and/or familial clinical association. In "met-

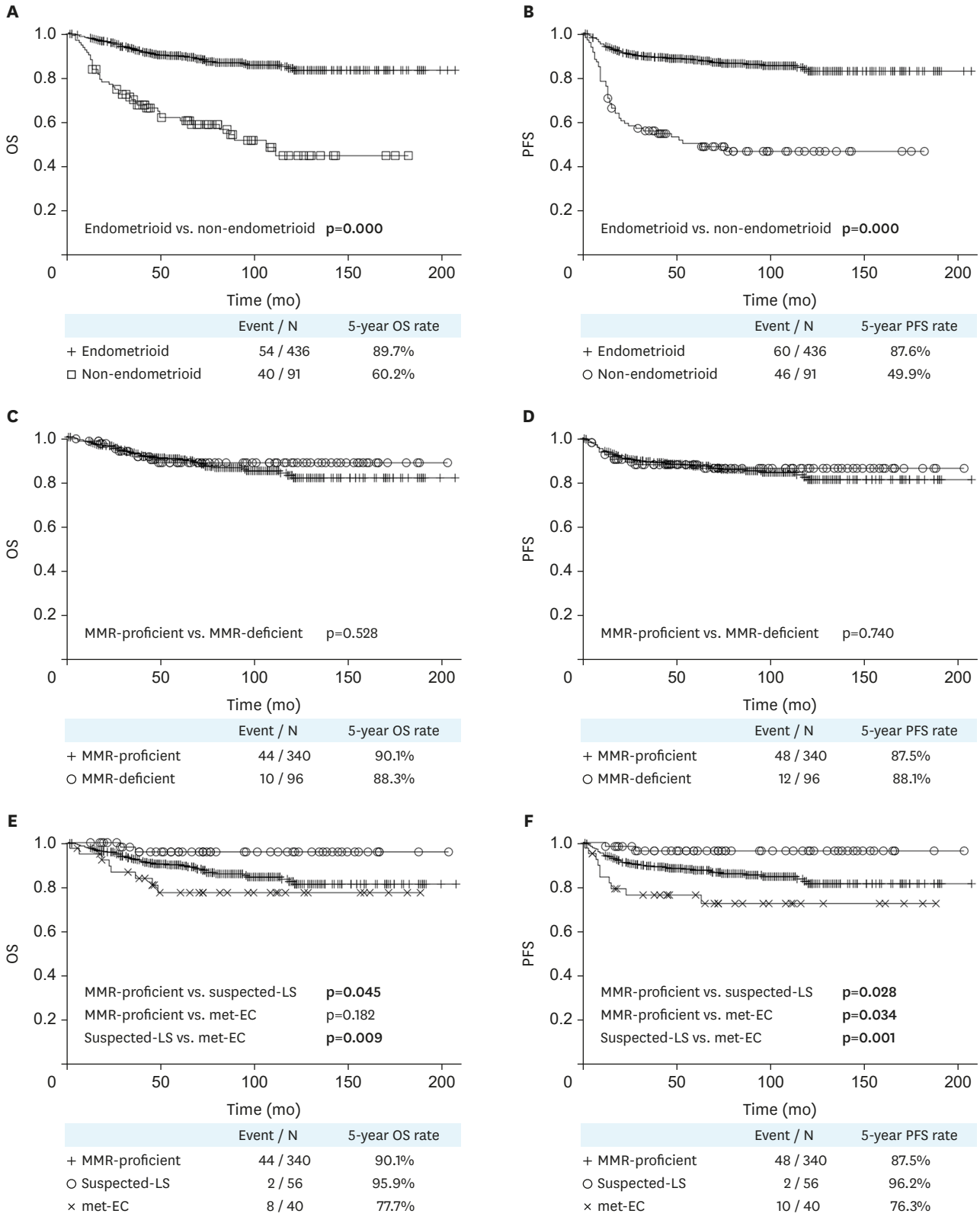

Fig. 4. Survival analysis by histological type. (A, B) OS and PFS by histological type. (C, D) OS and PFS in endometrioid type by MMR-status. (E, F) OS and PFS in endometrioid type.
LS, Lynch syndrome; met-EC, endometrial carcinomas with *MLH1* promoter hypermethylation; MMR, mismatch repair; OS, overall survival; PFS, progression-free survival.

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EC" cases, the risk of metachronous carcinogenesis has been reported to be comparable to that in "MMR-proficient" cases [24]. In this study, the personal CRC incidence and the prevalence in family history of CRC in the "met-EC" group were significantly lower than that in "suspected-LS" and were comparable to that in the "MMR-proficient" group. Patients from both "suspected-LS" and "met-EC" groups develop cancer through the inactivation of MMR genes, but the "met-EC" group shows little genetic effect because its inactivation is triggered by acquired DNA modification. Cases of MLH1 epimutation have been reported [25,26], but are very rare. The "met-EC" group would differ from the "suspected-LS" group not only in carcinogenic triggers but also in hereditary characteristics.

MMR-D has been reported to be a good prognostic factor in ECs [6,27]. In this study, patients with MMR-D showed significantly better OS and PFS than "MMR-proficient" patients. Shikama et al. [7] showed that the OS of the "suspected-LS" group, excluding ECs with *MLH1*-PHM from MMR-D cases, is excellent. "Met-EC" cases have been reported to have several poor prognostic factors [8,28,29], but few reports have detailed the long-term prognosis of these cases [8,9]. In this study, "met-EC" cases had a significantly lower proportion of grade 1 tumors and a higher proportion of advanced tumors than the other groups, and showed significantly worse OS and PFS than "suspected-LS" cases. Considering the multivariate analysis (**Table S1**) for prognostic factors, *MLH1*-PHM status was likely to influence tumor differentiation and progression.

Histological type is one of the most important prognostic factors in EC, and MMR-D is considered to be a favorable prognostic factor. When the endometrioid type cases in this study were analyzed, OS and PFS had no difference by MMR-status. It was particularly noteworthy in the endometrioid type that "met-EC" showed poorer prognosis than "MMR-proficient" or "suspected-LS" (**Fig. 4E and F**). Hence, *MLH1* promoter methylation analysis could reveal the poor prognosis group in endometrioid type cases with MMR-D.

Anti-PD-1 antibody, an immune checkpoint inhibitor, has been reported to show a high antitumor effect against MMR-D cancers [1]. EC with MMR-D is a candidate target tumor/cancer for anti-PD-1 antibody. EC patients with "suspected-LS" have an excellent prognosis with conventional therapies [7,29], so the application of anti-PD-1 antibodies would be rare. Among ECs with MMR-D, the "met-EC" group includes most of the advanced cases and has a high recurrence rate, so it would be the main target for anti-PD-1 antibody.

Tumor PD-L1 expression is considered a biomarker that predicts the effects of anti-PD-1 antibodies in some cancers [30]. The KEYNOTE-028 study showed the antitumor effect of pembrolizumab on PD-L1 positive ECs [31]. Susceptibility to the anti-PD-1 antibody for tumors with *MLH1*-PHM has not been reported, and the consensus on the association between ECs with *MLH1*-PHM and PD-L1 expression is immature [9,32]. In this retrospective study, the expression of PD-L1 in EC tumors could not be confirmed because of decreased antigenicity over time. The effect of anti-PD-1 antibody on "met-EC" cases should be individually verified in clinical practice.

MLH1 promoter methylation analysis would play a valuable role not only as a LS screening method but also as a clinical biomarker. This study's results would contribute to predicting the possibility for LS and Lynch-like cases, the prognosis of EC patients, and clinical susceptibility to anti-PD-1 antibodies. However, some limitations exist in this study. First, the therapeutic application of anti-PD-1 antibody to ECs does not occur on a large scale globally,

and its clinical effects are still being verified. Second, as not all the "suspected-LS" cases had undergone genetic testing, we could not directly compare "met-EC" cases with previously classified LS or Lynch-like cases [14].

In conclusion, "met-EC" cases are a subgroup with a poorer prognosis compared with "suspected-LS" cases. Cases with *MLH1*-PHM are the main target group for anti-PD-1 antibodies, and their clinical susceptibility should be verified individually.

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SUPPLEMENTARY MATERIALS

Table S1

Multivariate analysis for OS and PFS

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Fig. S1

Survival analysis by grade. (A, B) OS and PFS in low grade by MMR-status. (C, D) OS and PFS in low grade. (E, F) OS and PFS in high grade by MMR-status. (G, H) OS and PFS in high grade. Low grade included endometrioid G1 and G2, high grade included endometrioid G3.

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