Isolated loss of PMS2 immunohistochemical expression is frequently caused by heterogeneous *MLH1* promoter hypermethylation in Lynch syndrome screening for endometrial cancer patients

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ABSTRACT

Lynch syndrome (LS) is an autosomal dominant inherited disorder mainly caused by a germline mutation in the DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, *PMS2)* and is associated with increased risk of various cancers, particularly colorectal cancer and endometrial cancer (EC). Women with LS account for 2–6% of EC patients; it is clinically important to identify LS in such individuals for predicting and/or preventing additional LS-associated cancers. *PMS2* germline mutation (PMS2-LS) is the rarest contribution to LS etiology among the four LS-associated MMR germline mutations, and its detection is complicated. Therefore, prudent screening for PMS2-LS is important as it leads to an efficient LS-identification strategy. Immunohistochemistry (IHC) is recommended as a screening method for LS in EC. Isolated loss of PMS2 expression (IL-PMS2) is caused not only by PMS2-LS but also by *MLH1* germline mutation or *MLH1* promoter hypermethylation (MLH-PHM). This study aimed to determine the association between MLH1-PHM and IL-PMS2 to avoid inappropriate genetic analysis. We performed *MLH1* methylation analysis and *MLH1/PMS2* germline mutation testing for the IL-PMS2 cases. By performing MMR-IHC for 360 unselected ECs, we selected eight (2.2%) cases as IL-PMS2. Heterogeneous MLH1 staining and MLH1-PHM were detected in 4/8 (50%) IL-PMS2 tumors. Out of five IL-PMS2 patients who underwent genetic analysis, one had *PMS2* germline mutation with normal MLH1 expression (without MLH1-PHM) and no *MLH1* germline mutation was detected. We suggest that *MLH1* promoter methylation analysis for IL-PMS2 EC should be performed to exclude sporadic cases prior to further *PMS2* genetic testing.

Key word: Lynch syndrome, endometrial cancer, PMS2, *MLH1* promoter hypermethylation, heterogeneous

Introduction

Among endometrial cancer (EC) patients, Lynch syndrome (LS) accounts for approximately 2–6% of cases.[1-5] LS is an autosomal dominant inherited syndrome mainly caused by germline mutations in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, and *PMS2*.[6] Mutation carriers have an increased lifetime risk of developing colorectal cancer (CRC, 40–80%), EC (33–61%), ovarian cancer (9–12%), and other LS-associated cancers.[7] Thus, it is clinically relevant to identify LS women among EC patients in order to predict and prevent the development of other LS-associated cancers. It would also provide blood relatives an opportunity for genetic analysis and surveillance for LS-associated cancers. Each of the four MMR germline mutations lead to distinct molecular pathologies,[8] and thus individuals carrying different mutations should not be regarded as suffering from the same disease. *PMS2* germline mutation is associated with later onset, weaker family history, and a lower risk of cancer than other MMR germline mutations.[9,10] Indeed, *PMS2* germline mutation is the rarest genetic alteration among the four LS-associated MMR germline mutations, and its detection is more complicated than that of other MMR germline mutations due to the presence of a large family of highly homologous *PMS2* pseudogenes.[11]

Immunohistochemistry (IHC) is recommended as a primary screen for LS in patients with newly diagnosed EC,[12,13] as it can rapidly detect loss of MMR protein expression. In predicting MMR germline mutation, the sensitivity of IHC using a panel of four MMR antibodies (against MLH1, MSH2, MSH6, and PMS2) is as high as that of microsatellite instability（MSI）testing,[13,14] which has been also used as a screening tool for LS. IHC is simple and fast, cost effective, and practical in many institutions. It can also be used to predict corresponding germline mutations, and is more suited for detection of *MSH6* germline mutation than MSI testing.[12,14] In general, the presence of nuclear staining in tumor cells is good evidence of retained MMR protein, even if it is focal and weak staining.[14] This has led to neglect of staining pattern interpretation, with the exception of cases that show complete absence of nuclear staining**.** However, variable staining patterns are very confusing to interpret, as they present as heterogeneous staining, weak staining, and cytoplasmic staining.[14-16] These variabilities

are commonly seen in MLH1, and some studies have reported that *MLH1* germline mutation may underlie weak MLH1 staining.[17]

The major reason for loss of MLH1 expression in sporadic cancers is *MLH1* promoter hypermethylation (MLH1-PHM).[2] This phenomenon is seen in 15–20% of CRCs and 20–30% of ECs.[18] Performing *MLH1* promoter methylation analysis in order to determine the cause of MLH1 loss would avoid unnecessary *MLH1* germline mutation testing. MLH1-PHM is unevenly distributed in tumors, and there are some reports that this correlates with heterogeneous MLH1/PMS2 staining.[15,19] Therefore MLH1-PHM can occasionally lead to unclear staining in IHC.[16]

MLH1 and PMS2 proteins form functional heterodimer complexes.[20] MLH1 is obligatory for PMS2 protein stability, and its dysfunction leads to degradation and/or loss of PMS2.[20] The converse is not true, because MLH1 can also bind to other MMR proteins.[20] On the other hand, some *MLH1* germline mutations induce only loss of PMS2 protein and yet MLH1 antigenicity is retained.[16,21,22] Thus, in cases of isolated loss of PMS2 expression (IL-PMS2), MLH1 disorders cannot be excluded.[21,22] Guidelines from The National Society of Genetic Counselors (NSGC) and the Collaborative Group of the Americas on Inherited Colorectal Cancer (CGA-ICC) recommend *MLH1* germline mutation testing in IL-PMS2 cases where *PMS2* germline mutations are absent.[13] The National Comprehensive Cancer Network (NCCN) guidelines list *PMS2* and *MLH1* germline mutations as plausible etiologies in IL-PMS2.[23] These guidelines (and some additional studies [21,22]) mention *MLH1* germline mutation in IL-PMS2; yet, few studies have investigated *MLH1* promoter methylation in IL-PMS2. Moreover, all of the previous studies focused on CRC, and there is no adequate consensus on the genetic alterations that predispose individuals to EC.

In an LS identification strategy that adopted universal MMR-IHC screening, "Lynch like (LL)" patients who had MMR-IHC deficiency without germline mutations formed a distinct subgroup.[24-26] In families of LL CRC patients, the incidence of CRC was lower

than that in families of confirmed LS cases, and higher than that in the families of sporadic cases.[27] From this trend, both unknown hereditary cancers and sporadic cancers are likely to be intermixed in the LL CRC group.[24-26] It has been suggested that LL CRC patients and their relatives should undergo the same management as LS patients.[28] However, little is known regarding the clinical features of LL patients in EC.

In a previous study, we proposed a screening strategy for LS in 360 newly diagnosed EC patients with lenient triage (original criteria), using selective IHC and optional *MLH1* promoter methylation analysis.[29] We performed IHC on samples from all 360 of these participants, and detected 10 cases (2.8%) of IL-PMS2. Most of them were accompanied by MLH1 IHC abnormalities (such as heterogeneous or weak staining). Based on these results and existing knowledge, we hypothesized that MLH1-PHM might exist in some IL-PMS2 cases. Clarifying the MLH1-PHM status in IL-PMS2 cases would avoid unnecessary genetic analysis; moreover, it would spare individuals and relatives from uncomfortable clinical diagnostic interventions. With this in mind, we designed the current study to determine the association between MLH1-PHM and IL-PMS2.

Materials and Methods

Study population and procedures (Figure 1)

A total of 360 EC patients who were diagnosed at Akita University Hospital between January 2003 and December 2013 were identified retrospectively. All of the patients were Asians living in Japan. The patients' clinical data such as age, personal medical history, and family history were collected from medical records. We designed criteria, named "APF criteria" (our original criteria for selection according to **A**ge of onset < 50 years and/or **P**ersonal/**F**amily history of Lynch-associated cancer), and applied it to unselected EC patients. The cases satisfying one or more of the three criteria were considered to meet our criteria. We performed MMR-IHC on the tumor of patients who met our criteria in our previous study[29]. Additional IHC was performed on the tumor of patients who did not meet our criteria in this study. Performing the *MLH1* methylation assay and MMR germline mutation testing on cases with IL-PMS2, we investigated the association between MLH1-PHM and IL-PMS2. All study participants provided written informed consent in the prescribed document. Institutional Review Board of Akita University approved our study design.

Immunohistochemical staining for DNA mismatch repair proteins (MMR-IHC)

MMR-IHC was performed on tumors of all 360 EC patients to assess MMR protein (MLH1, MSH2, MSH6, and PMS2) expression, according to standard procedure. An appropriate paraffin-embedded tissue was cut at 4μm. The tissue sections were deparaffinized in xylenes and rehydrated in graded alcohols. Subsequently, antigen retrieval was performed in 10 mmol/L Tris-EDTA buffer (pH 9.0) in a microwave oven for 20 min. These sections were allowed to cool at room temperature. Then, the primary anti-bodies were applied overnight at 4°C. The primary antibodies were MLH1 (clone ES05; dilution 1:50; Dako), 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 with the Envision kit (Dako). The slides were counterstained with hematoxylin. Adjacent normal endometrium and lymphocytes in the slides were used as an internal positive control. We judged the complete absence of nuclear staining in the tumor cells as loss of MMR protein expression.

MLH1 promoter methylation analysis

In all eight IL-PMS2 cases, we performed *MLH1* promoter methylation analysis. The tumor DNA was extracted from mapped formalin-fixed, paraffin-embedded tissue sections to provide tumor samples for the assay. The SALSA MS-MLPA kit ME011 mismatch repair genes (MMR) (MRC-Holland, Amsterdam, The Netherlands) was used to detect aberrant CpG island methylation in the promoter of MMR genes, including 5 probes for *MLH1*. The MS-MLPA assay was performed as described by the manufacturer. We focused on the promoter C region (probe 3) which provides the best correlation with MLH1 expression.[30] Based on a previous study associated with gene silencing,[31] the dichotomization threshold to distinguish hypermethylated versus non-methylated samples was set at 15%.

Germline genetic testing

Five out of eight IL-PMS2 cases underwent the genetic analysis for this study. Germline mutation testing of *MLH1* and *PMS2* was performed on genomic DNA isolated from peripheral-blood leucocytes. Detection of point mutations was conducted using exon-by-exon PCR and direct sequencing of the whole coding sequence in and intron-exon boundaries for each gene. Large rearrangements (deletions and/or insertions) in the MMR gene were screened by MLPA according to manufacturer protocols (SALSA MLPA kits P003, P008).

Results

Performing MMR-IHC, we finally identified eight (2.2%) cases as IL-PMS2 out of unselected 360 EC patients. We had originally recognized ten cases as IL-PMS2 in the previous report, but we excluded two cases with weak PMS2 expression from the IL-PMS2 in this inspection. All eight IL-PMS2 cases met the original (APF) criteria (Figure 1). The clinical and pathological characteristics of the IL-PMS2 cases are shown in Table 1. No cases of IL-PMS2 met the Amsterdam Criteria II, and three (37.5%) met the SGO 5-10% criteria. MLH1-PHM was detected in 4 (50%) out of 8 IL-PMS2 cases (Table 1). In four cases with MLH1-PHM, between 25% and 65% of the *MLH1* promoter C region was hypermethylated. All cases with MLH1-PHM were accompanied with MLH1 heterogeneous staining and cytoplasmic immunoreactivity.

Out of eight IL-PMS2 cases, *PMS2* and *MLH1* germline mutation tests were performed in five (63%) who donated blood samples. Out of five IL-PMS2 patients who underwent genetic testing, one had *PMS2* germline mutation with normal MLH1 expression (without MLH1-PHM) and no *MLH1* germline mutation was detected (Table 1).

Discussion

PMS2 germline mutation is a rare cause of LS in EC, and the risk of LS-associated cancers is considerably lower with this genetic lesion than with those in the other three MMR genes.[9,10] Thus, the benefits of screening for *PMS2* germline mutation carriers (PMS2-LS) are less clear than those obtained by screening for other MMR germline mutation carriers. For developing the best screening strategy for LS in EC, exclusion of non-PMS2-LS is critical in order to avoid unnecessary *PMS2* germline mutation testing.

In this study, we detected MLH1-PHM in half of the cases with IL-PMS2. In the MLH1-PHM cases, no *PMS2* or *MLH1* germline mutations were found, and we thus considered these as instances of sporadic EC. In cases without MLH1-PHM, one *PMS2* germline mutation was detected, but no *MLH1* germline mutations were found. In all MLH1-PHM cases, MLH1 expression was heterogeneous. In contrast, we did not observe heterogeneous MLH1 staining in non-MLH1-PHM cases (Tables 1, 2).

Previous studies have focused on areas with heterogeneous MLH1 and PMS2 expression; areas with loss or retention of MLH1/PMS2 expression were assessed for *MLH1* promoter methylation separately.[15,19] Pai et al. described 6 cases of heterogeneous MLH1/PMS2 staining in EC.[15] MLH1-PHM was detected in all of these cases, and focal MLH1-PHM (limited to the areas with MLH1/PMS2 loss) was reported in two cases.[15] Joost et al. reported three cases of heterogeneous MLH1/PMS2 expression in CRC and performed methylation analysis in two of these cases.[19] Both cases showed MLH1-PHM in only the area with loss of MLH1/PMS2 expression.[19] These reports indicate that the heterogeneous MLH1/PMS2 expression was most likely attributable to MLH1-PHM. In our study, heterogeneous expression was detected only in MLH1 IHC; this expression pattern suggests that nonuniform hypermethylation was

present. There were two patterns in MLH 1 heterogeneous staining, that is, "compartmental," which was defined as retained/lost staining in large areas of the tumor, and "clonal," which was defined as retained/lost staining in whole glands or groups of glands (Table 2, Figure 2). Joost et al. also identified these patterns and suggested that they may be attributed to multiple causes, including variable epitope expression, second hit mutation or methylation in select tumors, or the influence of conditions in the tumor microenvironment, such as hypoxia and oxidative stress.[19] Additional studies are required to fully determine the meaning of the heterogeneous staining pattern.

In the case with *PMS2* germline mutation in our study, MLH1 expression in the tumor area was normal, whereas in the MLH1-PHM cases, MLH1 expression in the tumor area was heterogeneous. Further *PMS2* genetic testing could be avoided in IL-PMS2 cases with abnormal MLH1 expression patterns (such as heterogeneous and weak staining). Dudley et al. reported four *MLH1* germline mutations in 31 cases of IL-PMS2, and weak MLH1 staining was observed in two of those cases.[21] Per the recent reports summarized in Table 3, weak MLH1 staining, as revealed by IHC, has been observed in 20% (18 of 88) of cases where a *MLH1* germline mutation was present. Watson et al. reported *MLH1* germline mutation in cases with MLH1 heterogeneous staining.[32] Moreover, normal MLH1 staining was retained in 13% (11 of 88) of the *MLH1* germline mutation cases (Table 3). Therefore, MLH1 weak staining, heterogeneous staining, or even normal staining might be a result of false nuclear staining. In such cases, the possibility of *MLH1* germline mutation cannot be completely excluded. Shia et al. reported that weak MLH1 staining in IL-PMS2 cases may suggest *MLH1* genetic abnormalities.[16] This is because some pathogenic *MLH1* missense mutations functionally inactivate MLH1 protein and yet preserve its antigenicity.[16,33,34]

Cytoplasmic staining (CS) is one of the most confusing patterns associated with aberrant MLH1 expression in IHC. MLH1 CS was observed in all MLH1-PHM cases, and was sometimes seen locally in non-MLH1-PHM cases. In cases with MLH1 CS, it is challenging to determine whether MLH1 protein is completely absent. Shia et al. evaluated CS in CRC patients and found that CS extended to more than 30% of the tumor sample in 11% (12 of 105) of MLH1 IHC tests.[35] However, the presence of CS

was not correlated with MSI-H or germline mutation.[35] There are many difficulties associated with the interpretation of MLH1-IHC; these include confounding variables such as *MLH1* germline mutation, MLH1-PHM, CS, and other non-specific reactions. We suggest that IL-PMS2 cases should include not only PMS2-LS, but also MLH1-LS and MLH1-PHM subtypes.

On performing *MLH1* promoter methylation analysis to exclude sporadic cases, the following types of LS might go undetected: those in which *MLH1* germline mutation coexists with MLH1-PHM,[34] those with coexisting *PMS2* germline mutation and MLH1-PHM,[10] and those with autosomal dominant inherited MLH1-PHM (also known as constitutional MLH1 epimutation).[36] These cases are rare, but their identification is clinically significant, particularly if individuals have a strong family history and/or present with young onset of LS-associated cancers. Methylation analysis cannot completely confirm that tumors are sporadic. Thus, the first two types listed above can be excluded with a MMR germline mutation test, whereas autosomal dominant inherited MLH-1-PHM cannot.

MLH1 can interact with MLH3 or PMS1 instead of PMS2 to form a heterodimer that functionally compensates for the absence of MutLα (MLH1+PMS2), thereby delaying disease onset.[37] *MLH1* germline mutation tends to result in the typical form of LS, while *PMS2* germline mutation leads to an attenuated form of the disease.[37] The *MLH1* germline mutation–associated risk of CRC up to 70 years of age is considerably higher than the *PMS2* germline mutation–associated risk (40–80% and 15–20%, respectively).[10,38,39] Similarly, EC risk up to 70 years of age in individuals with *MLH1* germline mutation is higher than that of *PMS2* germline mutations (25–60% and 15%, respectively).[10,38,39] NCCN guidelines recommend separate surveillance for *MLH1* and *PMS2* germline mutation carriers.[23] Thus, verification of *MLH1* and *PMS2* germline mutation is important in the surveillance for individuals and their relatives. In the five IL-PMS2 cases in the current study, we did not find *MLH1* mutation carriers. However, according to previous reports, *MLH1* germline mutation was identified in 23–25% of IL-PMS2 cases.[21,22] When PMS2 expression is absent, the possibility of a *MLH1* germline mutation should not be excluded without additional information. This is true independent of the MLH1 expression status.

The spread of universal MMR-IHC screening for LS in EC would identify more LL (as well as LS) patients than classical selective screening.[26] Buchanan et al reviewed LL cases and reported that 52% (52/101) of MMR deficient EC cases were classified as LL.[26] MMR-IHC deficiency in LL tumors is due to unidentified germline MMR gene mutations, biallelic somatic gene inactivation, and other rare causes.[26] Haraldsdittir et al reported almost 70% of LL tumors had somatic mutations in MMR gene, and majority of LL cases were considered as nonhereditary.[40] To distinguish between LL tumor and sporadic EC may have considerable influence on the management of the LL patients and the relatives.

In the current study, we showed that 57% (4/7) of IL-PMS2 cases were misclassified as LL, and this error could be corrected by incorporating *MLH1* promoter methylation test.

In conclusion, we found that 50% of IL-PMS2 EC patients had MLH1-PHM. These MLH1-PHM cases did not have MMR germline mutation and were thus determined to be sporadic EC. *MLH1* promoter methylation analysis for IL-PMS2 EC should be performed to exclude sporadic cases prior to further *PMS2* genetic testing.

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FIGURE 1. Summary of this study. The *MLH1* promoter methylation test and germline mutation test for *MLH1* and *PMS2* were performed for isolated loss of PMS2 cases.

APF criteria, our original criteria for selection according to **A**ge of onset < 50 years and/or **P**ersonal/**F**amily history of Lynch-associated cancer.

IHC, immunohistochemistry analysis for MLH1, MSH2, MSH6, and PMS2.

ND, not done germline mutation test.

FIGURE 2. Examples of IHC staining for MMR protein. A, MLH1 heterogeneous staining (clonal loss) in case 5. (×10, ×40) B, Normal MSH2 staining in case 5. (×10) C, Complete loss of PMS2 staining in case 5. (×10) D, Normal MSH6 staining in case 5. (×10) E, MLH1 staining with cytoplasmic staining in case 6. (×10, ×40) F, MLH1 heterogeneous staining (compartment loss) in case 8. (×10, ×40)

TABLE 1. Clinical and Molecular Feature of Cases with Isolated Loss of PMS2 Expression

Case	Age (y)	Histologic Subtype	Grade	FIGO Stage	Criteria		*MLH1* Promoter Methylation Test	Germline Mutation Test
					AC	SGO 5-10%		
1	59	Endometrioid	1	IA	No	No	-	c.1972 C>T (*PMS2*)
2	63	Clear Cell	NA	IA	No	No	-	ND
3	63	Endometrioid	1	IB	No	No	-	ND
4	63	Endometrioid	2	IA	No	Yes	-	No mutation in *MLH1/PMS2*
5	48	Endometrioid	1	IA	No	Yes	+	No mutation in *MLH1/PMS2*
6	65	Endometrioid	2	IA	No	No	+	No mutation in *MLH1/PMS2*
7	61	Endometrioid	1	II	No	Yes	+	No mutation in *MLH1/PMS2*
8	77	Endometrioid	3	IA	No	No	+	ND

AC, Amsterdam Criteria II; SGO, Society of Gynecologic Oncologists; NA, not analyzed; ND, not done

-, hypermethylation (-); +, hypermethylation (+)

TABLE 2**.** Staining Patterns for MMR Proteins with Isolated Loss of PMS2 cases.

	MLH1			
Case	Nuclear staining	Cytoplasmic staining	MSH2	MSH6
1*	Strong uniform	P	Partial weak	Partial heterogeneous
2	Strong uniform	N	Normal	Normal
3	Weak in ~ 50%	N	Partial weak	Partial weak
4	Strong uniform	N	Normal	Normal
5	Heterogeneous (Clonal loss in > 50%)	P	Partial weak	Partial weak
6	Heterogeneous (Compartment loss in > 50%)	P	Partial weak	Partial weak
7	Heterogeneous (Compartment loss in ~ 50%)	P	Partial weak	Partial weak
8	Heterogeneous (Compartment loss in > 50%)	P	Partial weak	Normal

CS, cytoplasmic staining; P, positive staining; N, negative staining

*Known *PMS2* germline mutation

TABLE 3. False Staining for MLH1 According to *MLH1* Germline Mutation.

Reference	*MLH1* Germline Mutation	MLH1 False Staining	MLH1 staining pattern	
			Weak Staining	Normal Staining
Mangold et al[17]	44	15	14	1
Shia et al[27]	9	5	1	4
Jong et al[22]	35	9	3	6
Total	88	29	18	11

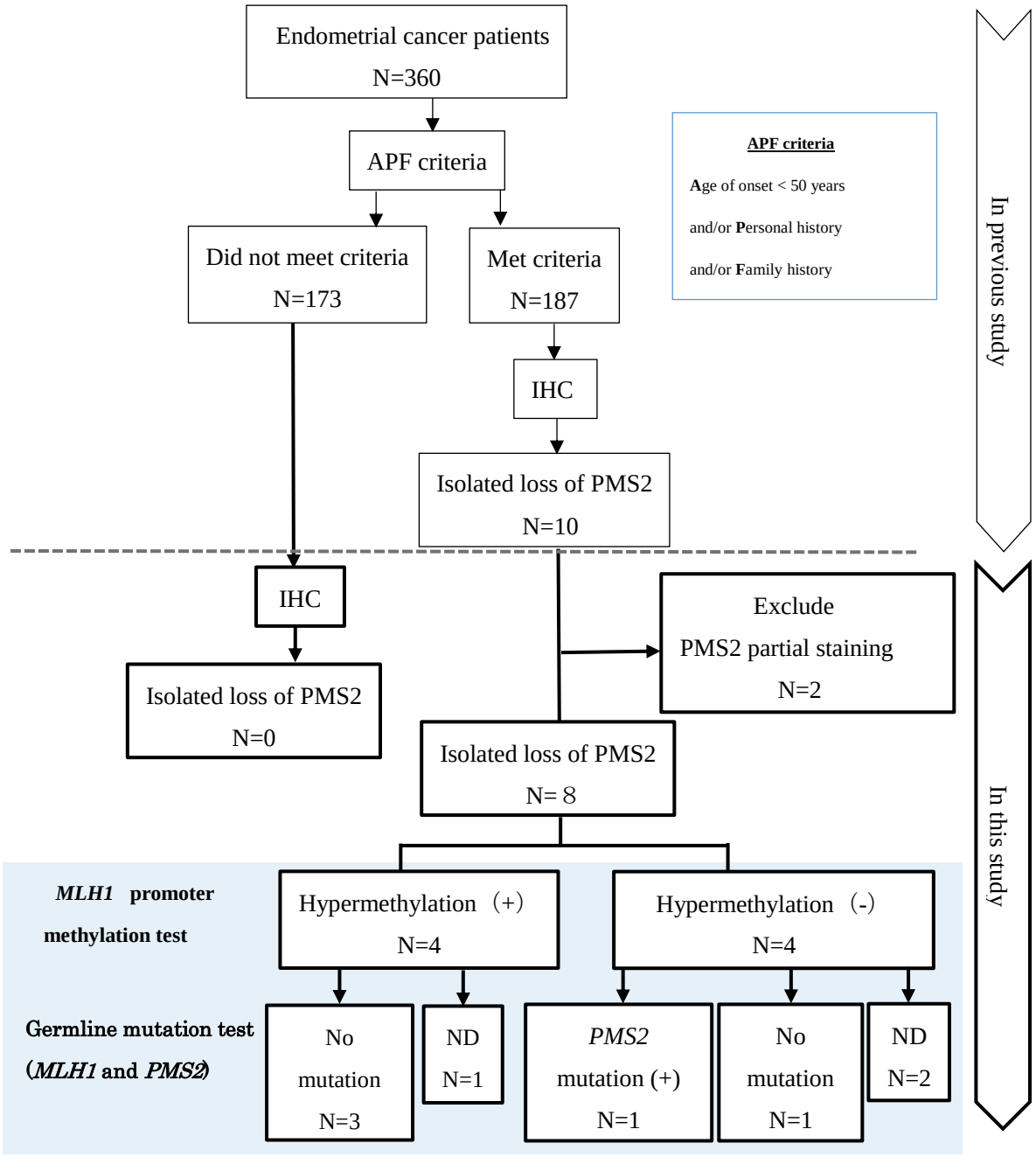
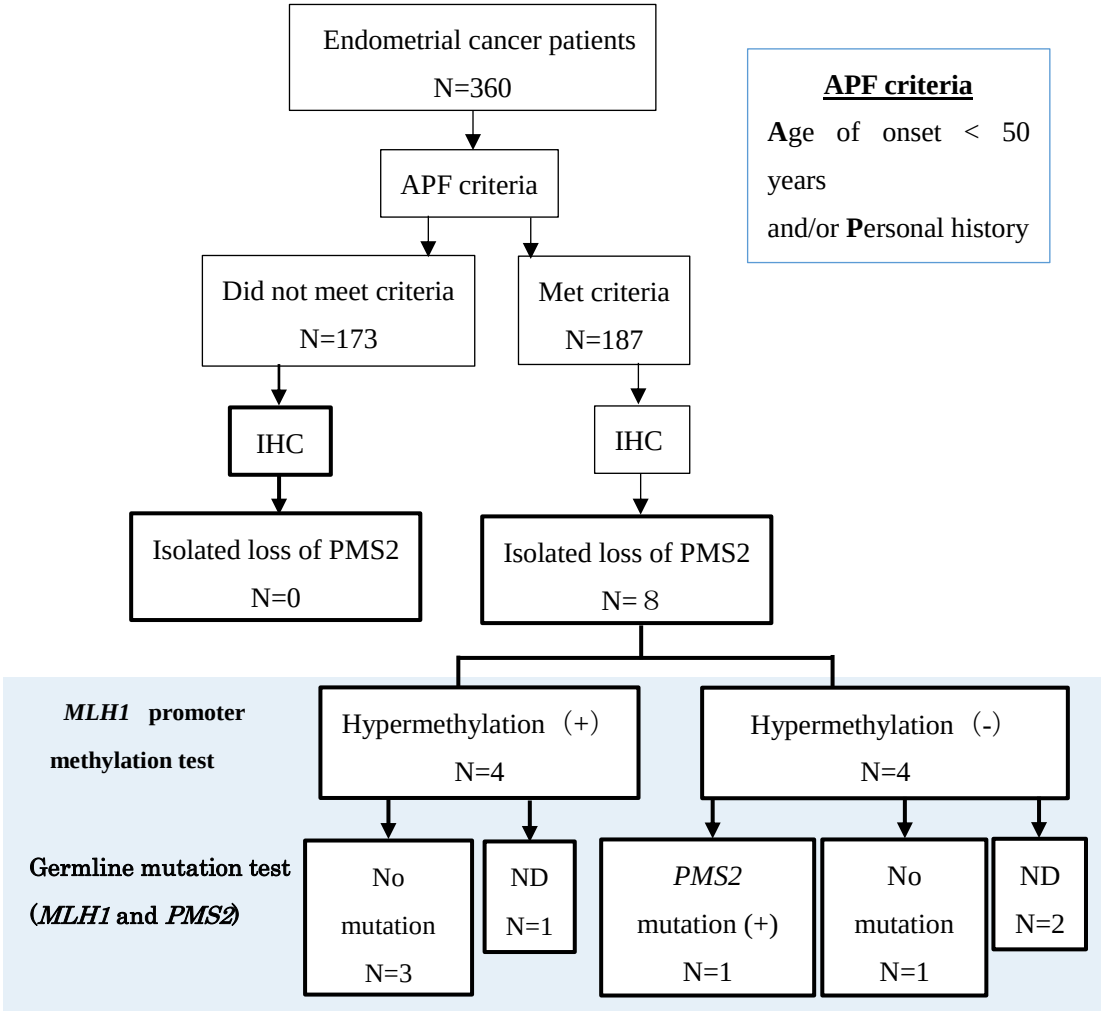


FIGURE 1. Summary of this study. The *MLH1* promoter methylation test and germline mutation test for *MLH1* and *PMS2* were performed for isolated loss of PMS2 cases.

APF criteria, our original criteria for selection according to **A**ge of onset < 50 years and/or **P**ersonal/**F**amily history of Lynch-associated cancer.

IHC, immunohistochemistry analysis for MLH1, MSH2, MSH6, and PMS2.

ND, not done germline mutation test.


Endometrial cancer patients
N=360
APF criteria
APF criteria
Age of onset < 50 years
and/or Personal history
Did not meet criteria
N=173
Met criteria
N=187
IHC
IHC
Isolated loss of PMS2
N=0
Isolated loss of PMS2
N= 8
MLH1 promoter methylation test
Hypermethylation (+)
N=4
Hypermethylation (-)
N=4
Germline mutation test (MLH1 and PMS2)
No mutation
N=3
ND
N=1
PMS2 mutation (+)
N=1
No mutation
N=1
ND
N=2

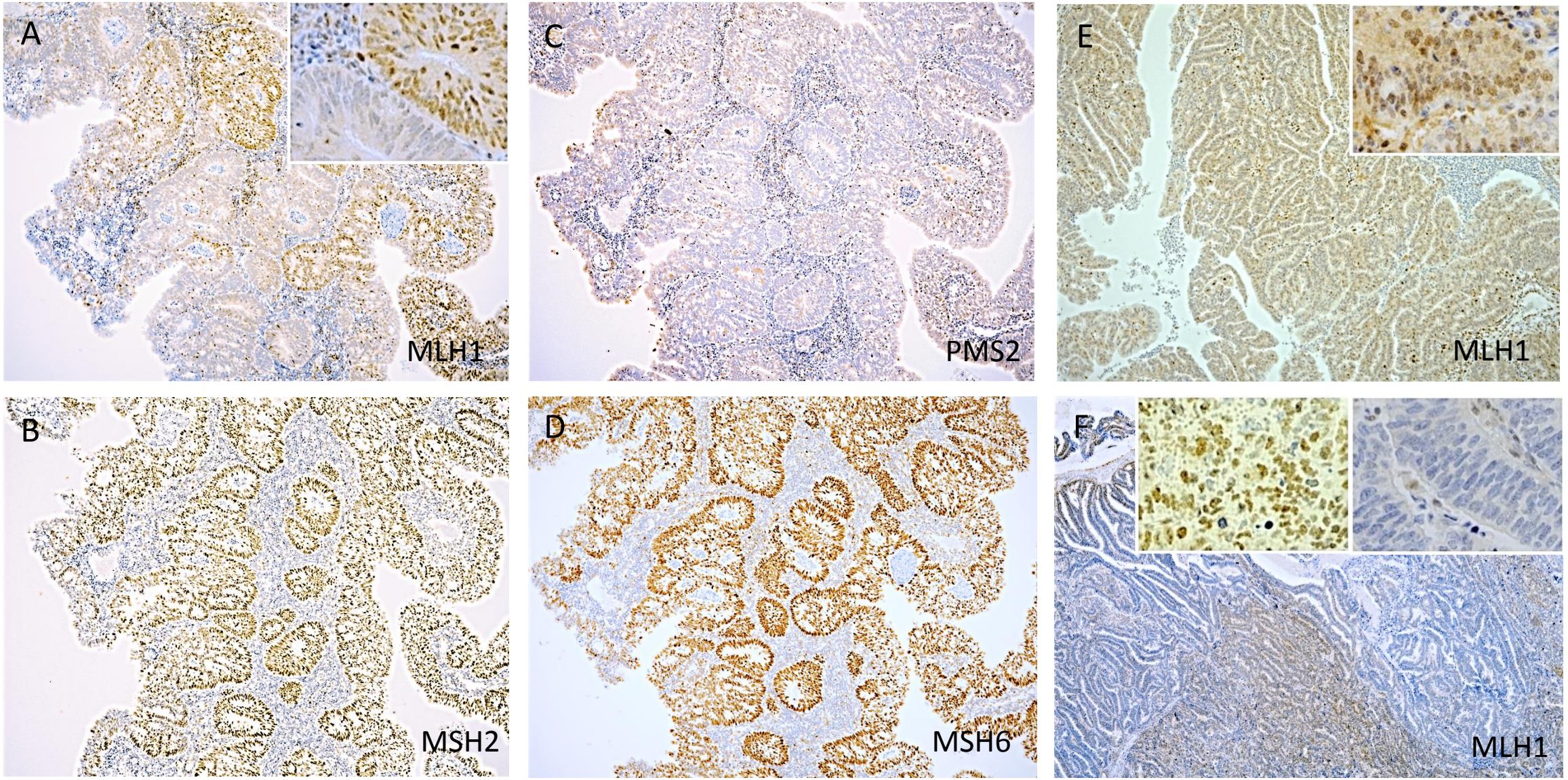


FIGURE 2. Examples of IHC staining for MMR protein. A, MLH1 heterogeneous staining (clonal loss) in case 5. (×10, ×40) B, Normal MSH2 staining in case 5. (×10) C, Complete loss of PMS2 staining in case 5. (×10) D, Normal MSH6 staining in case 5. (×10) E, MLH1 staining with cytoplasmic staining in case 6. (×10, ×40) F, MLH1 heterogeneous staining (compartment loss) in case 8. (×10, ×40)