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Molecular Analysis of Endometrial Tumorigenesis: Importance of Complex Hyperplasia Regardless of Atypia

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Abstract

Purpose: Endometrial carcinoma (EC) is common in the population and the most frequent extracolonic malignancy in hereditary nonpolyposis colorectal carcinoma (HNPCC)/Lynch syndrome. We characterized precursor lesions of endometrioid EC to identify markers of malignant transformation and tumor progression.

Experimental Design: Serial specimens of normal endometrium, simple hyperplasia, complex hyperplasia without atypia, complex hyperplasia with atypia, and endometrial carcinoma obtained during a 10-year surveillance of DNA mismatch repair (MMR) gene mutation carriers (together 110 samples) were molecularly profiled and compared with a sporadic reference series of endometrial specimens taken for nonmalignant reasons (62 samples).

Results: Among MMR gene mutation carriers, decreased MMR protein expression was present in 7% in normal endometrium, 40% in simple hyperplasia, 100% in complex hyperplasia without atypia, 92% in complex hyperplasia with atypia, and 100% in endometrial carcinoma. Microsatellite instability frequencies were lower (6%, 17%, 67%, 38%, and 64%, respectively). Among 24 tumor suppressor genes, the number of methylated loci increased from normal endometrium to simple hyperplasia to complex hyperplasia (complex hyperplasia without atypia/complex hyperplasia with atypia) in both Lynch syndrome and reference series. The most frequently methylated genes were *CDH13, RASSF1A*, and *GSTP1*. In MMR gene mutation carriers, MMR and methylation defects appeared up to 12 years before endometrial carcinoma.

Conclusions: Molecular changes in endometrial tissue are detectable several years before endometrial carcinoma in genetically predisposed individuals. Abnormal MMR and methylation classify normal endometrium and simple hyperplasia into one category and complex hyperplasia without atypia, complex hyperplasia with atypia, and endometrial carcinoma into another, suggesting that, contrary to a traditional view, complex hyperplasia without atypia and complex hyperplasia with atypia are equally important as precursor lesions of endometrial carcinoma. (Clin Cancer Res 2009;15(18):5772–83)

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Endometrial carcinoma is the most common malignant tumor of the female genital organs in industrialized countries (1) and comprises 4% of all cancers in women worldwide (2). Estrogen-dependent tumors (type I, endometrioid endometrial carcinomas) account for 80% to 85% of cases and non-estrogen-dependent (type II, nonendometrioid endometrial carcinomas) account for the rest. Type I tumors occur in premenopausal and younger postmenopausal women, are typically low-grade and localized, and have a favorable outcome, whereas type II tumors occur in older postmenopausal women, are of high grade and more advanced stage, and have worse prognosis (1, 2). Type I tumors are considered to form in a background of endometrial hyperplasia. The WHO classifies endometrial hyperplasias as simple or complex, by degree of architectural abnormality, and as having or not having atypia, by cytology (3). Epidemiologic studies suggest that the risk of progression to malignancy

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Translational Relevance

Endometrial carcinoma is a common malignancy in the general population and in Lynch syndrome associated with inherited mismatch repair (MMR) deficiency. In an attempt to identify markers of malignant transformation and tumor progression, endometrial hyperplasias, precursor lesions of endometrioid endometrial carcinoma, were molecularly characterized. In both Lynch syndrome and sporadic cases, tumor suppressor promoter hypermethylation distinguished complex hyperplasias irrespective of atypia, resembling endometrial carcinoma and contrasting with simple hyperplasia and normal endometrium. In Lynch syndrome, absent MMR protein expression and microsatellite instability paralleled these findings, and molecular changes were detectable several years before endometrial carcinoma. Our findings challenge a traditional view of nuclear atypia being critical for progression to malignancy. Molecular alterations in biopsy specimens could serve as an indicator for prophylactic hysterectomy especially in high-risk individuals, for example, MMR mutation carriers who would not otherwise opt for prophylactic surgery.

is low (≤3%) for hyperplasia without atypia (simple hyperplasia, complex hyperplasia without atypia, and complex hyperplasia), whereas atypia is associated with a significant risk of carcinoma, 8% for simple hyperplasia (simple atypical hyperplasia) and 29% for complex hyperplasia (complex hyperplasia with atypia and complex atypical hyperplasia; ref. 4).

Approximately 5% of endometrial carcinomas are familial, and many of these represent hereditary nonpolyposis colorectal carcinoma/Lynch syndrome (5). In this multiorgan cancer syndrome associated with inherited defects in DNA mismatch repair (MMR), endometrial carcinoma, which is usually of the endometrioid type (type I), is the most common extracolonic malignancy (6). Lifetime risk of endometrial carcinoma varies between 32% and 60% in Lynch syndrome compared with 1% in the general population (6–8). Although endometrial carcinoma is generally thought to develop via precursor lesions along with the increasing accumulation of molecular genetic-changes, in analogy to colorectal cancer, steps that lead to endometrial carcinoma are less well understood. Alterations associated with type I endometrial carcinoma include microsatellite instability (MSI; 25-30%), *PTEN* inactivation (up to 60%), *KRAS* mutations (10-30%), β-catenin gene mutations (25-40%), and epigenetic changes such as tumor suppressor promoter methylation (variable percentages; refs. 9–11). Because Lynch syndrome is a disease of progression with accelerated tumorigenesis (12), the syndrome offers a useful model to dissect the molecular steps involved in endometrial carcinoma development.

We took advantage of serial endometrial biopsy samples taken during a 10-year follow-up of Lynch syndrome mutation carriers (13). That study found that endometrial aspiration biopsy (Pipelle) was the most efficient method to find atypical hyperplasia and/or cancer. Our first aim was to study the molecular changes that accompany endometrial tumorigenesis in Lynch syndrome and, in particular, to find out if molecular alterations appear before histologic malignancy. The second aim was to compare the various types of hyperplasia in Lynch syndrome versus sporadic cases to evaluate the extent of similarity in tumorigenic steps between these.

Materials and Methods

Patients and samples. This study was based on female MMR gene mutation carriers enrolled in clinical surveillance for endometrial carcinoma (13). Among cases screened at two major participating hospitals (Jyväskylä Central Hospital and Helsinki University Central Hospital) during 1996 to 2005, all who developed endometrial hyperplasia or endometrial carcinoma and from which successive endometrial aspiration biopsy (Pipelle) or hysterectomy specimens were available were included ("core" series). For DNA-based analyses (MSI and methylation), samples from other participating Finnish hospitals as well as specimens obtained after 2005 were also available and formed an "extended" series when added to the core series (110 samples from 54 individuals in total; Table 1). For practical reasons, the mutation-positive series was divided into group I, with the development of endometrial carcinoma as the endpoint, and group II who did not develop endometrial carcinoma during follow-up. Time in surveillance was calculated from the date of enrollment into surveillance to a relevant endpoint, which could be endometrial carcinoma diagnosis (group I), hysterectomy (groups I and II), or the most recent observation of not having developed any cancer (group II). Time in surveillance was 0.5 to 11 years (mean, 5.9 years) for group I and 1 to 11 years (mean, 4.8 years) for group II. The distribution of germ-line mutations (*MLH1*, *MSH2*, and

Table 1. Number of available samples and average ages (in years) in which specimens were taken for groups I to III

Histology	Group I (mutation positive, endometrial carcinoma positive)		Group II (mutation positive, endometrial carcinoma negative)		Group III (sporadic, mutation negative, endometrial carcinoma negative)		Total	
	No.	Age	No.	Age	No.	Age	No.	Age
Normal	13	42.0	37	45.4	21	48.9	71	45.4
Simple hyperplasia	3	46.7	8	48.4	11	47.1	22	47.4
Complex hyperplasia without atypia	3	46.7	7	50.4	14	61.0	24	52.7
Complex hyperplasia with atypia	10	48.7	15	47.9	16	61.0	41	52.5
Endometrial carcinoma	14	50.3	—	—	—	—	14	50.3
Total	43	46.9	67	48.0	62	54.5	172	49.9

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Table 2. Frequencies of MSI, decreased MMR protein expression, and tumor suppressor promoter methylation in groups I to III

	Group I					Group II			
	Normal	**Simple hyperplasia**	**Complex hyperplasia without atypia**	**Complex hyperplasia with atypia**	**Endometrial carcinoma**	**Normal**	**Simple hyperplasia**	**Complex hyperplasia without atypia**	**Complex hyperplasia with atypia**
				"Core" series					
Proportion with unstable marker (%)									
0	10/10 (100)	2/3 (67)	0/2 (0)	5/8 (62)	4/11 (36)	24/26 (92)	3/3 (100)	1/1 (100)	3/5 (60)
1	0/10 (0)	1/3 (33)	0/2 (0)	0/8 (0)	4/11 (36)	2/26 (8)	0/3 (0)	0/1 (0)	1/5 (20)
2-3	0/10 (0)	0/3 (0)	2/2 (100)	3/8 (38)	3/11 (28)	0/26 (0)	0/3 (0)	0/1 (0)	1/5 (20)
Frequency of MSI defined as at least one unstable marker (%)	0/10 (0)	1/3 (33)	2/2 (100)	3/8 (38)	7/11 (64)	2/26 (8)	0/3 (0)	0/1 (0)	2/5 (40)
Proportion (%) with decreased MMR protein corresponding to germ-line mutation	0/8 (0)	2/3 (67)	2/2 (100)	7/7 (100)	11/11 (100)	2/21 (10)	0/2 (0)	1/1 (100)	4/5 (80)
				"Extended" series					
Frequency of MSI defined as at least one unstable marker (%)	0/13 (0)	1/3 (33)	3/3 (100)	6/10 (60)	8/12 (67)	2/37 (5)	3/8 (38)	4/7 (57)	11/15 (73)
Average no. tumor suppressor genes with promoter methylation (out of 24)/ sample	0.3	1.0	4.0	2.1	2.1	1.5	2.3	2.8	2.6

MSH6) was 73%, 13%, and 13% in group I (15 individuals) and 97%, 3%, and 0% in group II (39 individuals). Endometrial samples from consecutive sporadic cases, not under surveillance and undergoing endometrial biopsy or surgery for nonmalignant reasons, were used for reference (group III in Table 1, 62 samples from equally many individuals).

Samples of normal endometrium (63% proliferatory and 37% secretory), hyperplasia (simple hyperplasia, complex hyperplasia without atypia, and complex hyperplasia with atypia), and endometrial carcinoma were included (the rare category of simple atypical hyperplasia was not represented). Histologic diagnoses were based on evaluation by two different pathologists in each case. The mean ages at which samples were taken were 46.9 years (range, 31-71 years) in group I, 48.0 years (range, 34-56 years) in group II, and 54.5 years (range, 40-79 years) in group III. In group I, all endometrial carcinomas, except one (case 3 who had papillary serous carcinoma), represented endometrioid adenocarcinoma. Among 14 endometrial carcinoma tumors, the grade was 1 in 43%, 2 in 50%, and 3 in 7%. Most had localized disease, the stage being I in 86%, II in 7%, and III in 7%, according to the Federation of Gynecology and Obstetrics classification (14).

Formalin-fixed, paraffin-embedded tissue samples were collected from pathology departments, sectioned, and used for DNA extraction (15) or immunohistochemical analysis. For DNA extraction, the specimens were manually microdissected to separate areas of normal, hyperplasia, and tumor tissue whenever possible. In endometrial carcinoma samples, the average percentage of tumor cells was 60.5% (range, 30-90%). All human specimens were obtained after informed consent and approval from the review boards of the Helsinki University Central Hospital and Jyväskylä Central Hospital.

Immunohistochemical analysis. We used the following mouse primary antibodies: anti-MLH1 (clone G168-15; Pharmingen), anti-MSH2 (clone FE-11; Calbiochem), and anti-MSH6 (clone 44; Transduction Laboratories). The DAKO EnVision+ System (DAKOCytomation) was used according to the manufacturer's instructions. Antigen retrieval was done in 1 mmol/L EDTA buffer (pH 8.0) in a microwave oven for 10 min. Results were interpreted as described (16).

MSI analysis. Mononucleotide repeat markers *BAT25* and *BAT26* and dinucleotide repeat marker *D5S346* were used from the Bethesda panel (17). Fragments were analyzed with ABI Prism GeneMapper 4.0 (Applied Biosystems) software program. Samples with at least one unstable marker were interpreted as having MSI.

Methylation-specific multiplex ligation–dependent probe amplification. SALSA methylation-specific multiplex ligation–dependent probe amplification (MS-MLPA) ME001B (MRC-Holland) probe mix, including 24 different tumor suppressor genes, was used. All reactions were carried out according to the manufacturer's instructions.[8] In this method, the probe contains a recognition sequence for the methylation-sensitive enzyme *Hha*I. Only templates with methylated *Hha*I sites, resistant to *Hha*I cleavage, generate a signal after probe ligation and

[8] http://www.mrc-holland.com
[9] http://faculty.vassar.edu/lowry/VassarStats.html

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Table 2. Frequencies of MSI, decreased MMR protein expression, and tumor suppressor promoter methylation in groups I to III (Cont'd)

Groups I and II (Lynch syndrome)				Group III (reference)			
Normal	**Simple hyperplasia**	**Complex hyperplasia without atypia**	**Complex hyperplasia with atypia**	**Normal**	**Simple hyperplasia**	**Complex hyperplasia without atypia**	**Complex hyperplasia with atypia**
"Core" series							
34/36 (94)	5/6 (83)	1/3 (33)	8/13 (61)	21/21 (100)	N/A	N/A	N/A
2/36 (6)	1/6 (17)	0/3 (0)	1/13 (7)	0/21 (0)	N/A	N/A	N/A
0/36 (0)	0/6 (0)	2/3 (67)	4/13 (32)	0/21 (0)	N/A	N/A	N/A
2/36 (6)	1/6 (17)	2/3 (67)	5/13 (38)	0/21 (0)	N/A	N/A	N/A
2/29 (7)	2/5 (40)	3/3 (100)	11/12 (92)	N/A	N/A	N/A	N/A
"Extended" series							
2/50 (4)	4/11 (36)	7/10 (70)	17/25 (68)				
1.1	2.0	3.1	2.4	0.3	0.6	2.4	2.7

NOTE: See Materials and Methods for "core" and "extended" series.
Abbreviation: N/A, not available.

amplification. Paraffin-extracted DNA (150-800 ng) was used for each MS-MLPA reaction. Amplification products were separated by capillary electrophoresis and analyzed with ABI Prism GeneMapper 4.0 (Applied Biosystems). In every assay, normal DNA derived from lymphocytes from healthy individuals was included as a control for the lack of methylation and tumor cell line (RKO) as a control for methylation (18). Methylation dosage ratios were calculated as described (18). A dosage ratio of 0.15 (corresponding to 15% of methylated DNA), which has given the best discrimination between normal and tumor DNA in our earlier studies (18–20), was regarded to indicate promoter methylation.

Statistical analysis. Programs from the VassarStats Web site[9] were used. Fisher's exact probability test was used to evaluate differences between groups in frequencies of MSI, abnormal immunohistochemistry, or methylation of individual genes. When analyzing differences in the average number of methylated genes per sample, *t* test for independent samples was applied. All reported *P* values were two-tailed and *P* values < 0.05 were considered significant.

Results

This retrospective study was conducted on serial specimens of endometrium with various histologic diagnoses, taken by aspiration biopsy or surgery during a 10-year surveillance of MMR gene mutation carriers (13), some of which developed (group I) and others did not develop (group II) endometrial carcinoma by the end of follow-up (see Materials and Methods and Table 1). Series of unrelated samples of normal endometrium and hyperplasia taken from consecutive sporadic cases undergoing endometrial biopsy or hysterectomy for nonmalignant reasons (group III) were used for comparison. Table 2 provides a summary of all molecular alterations observed in different groups.

DNA MMR status. Among MMR gene mutation carriers, results obtained from groups I and II were broadly comparable with each other (Table 2), which made it possible to combine these groups into a "Lynch syndrome group" for most subsequent analyses. In the Lynch syndrome group, decreased MMR protein expression corresponding to the germ-line mutation was observed in 2 of 29 (7%) in normal endometrium, 2 of 5 (40%) in simple hyperplasia, 3 of 3 (100%) in complex hyperplasia without atypia, 11 of 12 (92%) in complex hyperplasia with atypia, and 11 of 11 (100%) in endometrial carcinoma (Table 2). MSI frequencies in the same core series were 2 of 36 (6%), 1 of 6 (17%), 2 of 3 (67%), 5 of 13 (38%), and 7 of 11 (64%), respectively, suggesting that immunohistochemical detection of MMR protein expression was more sensitive than MSI analysis to identify MMR defects. Instability of mononucleotide repeats (*BAT25* and *BAT26*) accounted for most cases with MSI, and among those cases with one unstable marker specifically, *BAT26* was involved in 70% and *D5S346* in 30%. By histology, results from immunohistochemistry and MSI analysis indicated that normal endometrium was MMR proficient (with the exception of two cases), whereas MMR defects were observed in most cases of complex hyperplasia, whether complex hyperplasia without atypia or complex hyperplasia with atypia, and endometrial carcinoma. Differences between

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Table 3. Molecular alterations in endometrial samples taken at different time points from carriers of MMR gene germ-line mutations (groups I and II)

	Patient ID	Germ-line mutation	Endometrial histology	Time before last sampling	Protein expression by immunohistochemistry			No. unstable markers out of 3	Methylated genes out of 24
					MLH1	MSH2	MSH6		
Group I	1	*MLH1* del ex16	Normal	13 y	+	+	cytopl.+	0	None
			Normal	11 y	+	+	(+)	0	None
			Normal	6 y	+	+	+	0	None
			Endometrial carcinoma	0	-	+	+	2	*RASSF1, CDH13*
	2	*MLH1* del ex16	Normal	3 y	+	+	+	0	*APC, CDH13*
			+ Complex hyperplasia with atypia		-	(+)	+		
			Complex hyperplasia with atypia	1 mo	-	+	+	0	None
			+Normal		+	+	+		
			Endometrial carcinoma	0	-	+	+	0	*CDH13*
	3	*MLH1* del ex16	Normal	3 y	+	+	+	0	NA
			Simple hyperplasia	3 mo	+	+	+	0	*GSTP1*
			Endometrial carcinoma	0	-	+	+	0	None
	4	*MLH1* del ex16	Normal	3 y	+	+	+	0	*RASSF1*
			+ Complex hyperplasia with atypia		-	+	+		
			Endometrial carcinoma	0	-	+	+	0	*RASSF1, GSTP1, CDH13*
	5	*MSH6* c3938-3941 dupGTTA	Normal	12 y	+	(+)	+	0	*CHD13*
			Normal	2 y	+	+	+	0	None
			Endometrial carcinoma	0	+	-	NA	1	*CDKN2B, RASSF1, CDH13*
	7	*MLH1* R659X	Simple hyperplasia	0.5 mo	-	+	+	1	NA
			Complex hyperplasia with atypia	0.5 mo	+/-	+	NA	1	NA
			+ Simple hyperplasia		+/-	+	NA		
			Normal	0	+	+	+	0	NA
			Complex hyperplasia with atypia	0	-	+	+	2	NA
			Endometrial carcinoma	0	-	+/-	+	3	*PTEN, TP73, CDH13, GSTP1*
	8	*MLH1* del ex16	Normal	4 y	+	+	+	0	NA
			Normal	3 y	+	+	(+)	0	NA
			Simple hyperplasia	2 y	-	+	+	0	NA
			Complex hyperplasia with atypia	0	-	+	(+)	3	*RASSF1, CDH13*
			Endometrial carcinoma	0	-	+	+	2	*CDKN2A*
Group II	13	*MLH1* R659P	Endometritis chronica	8 y	+/-	+	+	0	*CDKN2B, CDH13, GSTP1*
			Complex hyperplasia with atypia	3 mo	-	+	+	2	*TIMP3, CD44, RASSF1, CDH13*

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Table 3. Molecular alterations in endometrial samples taken at different time points from carriers of MMR gene germ-line mutations (groups I and II) (Cont'd)

	Patient ID	Germ-line mutation	Endometrial histology	Time before last sampling	Protein expression by immunohistochemistry			No. unstable markers out of 3	Methylated genes out of 24
					MLH1	MSH2	MSH6		
Group II			Complex hyperplasia with atypia	0	-	-	-	1	*TIMP3, CDKN2A, PTEN, RASSF1, CDH13, GSTP1*
			+ Normal		+	+	+		
	14	*MLH1* R659P	Simple hyperplasia	1 y	+	+	+	0	*TIMP3, GSTP1*
			Normal	7 mo	+	+	+	0	*CDKN2B, CDH13*
			Normal	0	NA	NA	NA	0	None
	15	*MLH1* 454-1 g>a	Normal	2	NA	NA	NA	0	*CDH13*
			Simple hyperplasia	0	+	+	+	0	*ATM, CDKN2B, CDKN1B*
	20	*MLH1* del ex16	Normal	5 y	NA	NA	NA	0	*CDH13, GSTP1*
			Complex hyperplasia with atypia	0.5 mo	+	+	+	0	*APC, RASSF1, CDH13, GSTP1*
			Normal	0	-	+	+	0	None
			+ Complex hyperplasia with atypia		-	+	+		
	24	*MLH1* del ex16	Normal	6 y	+	+	+	0	*RARB, PTEN, ESR1, RASSF1*
			Simple hyperplasia	0	+	+	+	0	*RASSF1, CDH13*
			Complex hyperplasia without atypia	0	-	+	+	0	*RASSF1, CDH13*
	109	*MLH1*	Normal	2 mo				0	ND
			Normal	0				0	None
			Complex hyperplasia without atypia	2 mo				1	*FHIT, CDH13, GSTP1*
			Complex hyperplasia with atypia	0				0	*APC, CDH13*

Abbreviations: +, positive expression; -, negative expression; (+), weak positive expression; +/-, positive and negative expression.

normal endometrium versus complex hyperplasia without atypia + complex hyperplasia with atypia and normal endometrium versus endometrial carcinoma (but not normal endometrium versus simple hyperplasia) were statistically significant ($P < 0.01$) with respect to either MSI or abnormal MMR protein expression. The general trends for MSI between histologic categories persisted after the inclusion of additional cases ("extended" series; Table 2).

Temporal appearance of molecular genetic changes in endometrial tissues from MMR gene mutation carriers is depicted in Table 3 (only cases from which serial samples of different histologies were available are included). In group I, absent MMR protein was detectable in endometrial hyperplasia up to 3 years before the diagnosis of endometrial carcinoma (cases 2, 4, 7, and 8 in Table 3). Representative examples of immunohistochemistry and MSI results from simple hyperplasia, complex hyperplasia with atypia, and endometrial carcinoma specimens (originating from cases 8 and 24) are shown in Fig. 1.

In the reference group (group III), MSI was not observed at all in normal endometrium (Table 2). In regard to hyperplasia, *MLH1* promoter methylation was used as a surrogate marker for MMR status because it is known that most sporadic MMR-deficient endometrial carcinomas arise through promoter methylation (21, 22), and only cases with *MLH1* methylation ($n = 4$) were further examined for MMR protein expression and MSI. Two of 37 hyperplasia samples, one complex hyperplasia without atypia and one complex hyperplasia with atypia, had MLH1 protein loss and/or MSI (5%; data not shown).

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Tumor suppressor promoter methylation. Among 24 tumor suppressor genes examined for promoter methylation by MS-MLPA, the average number of methylated loci increased progressively from normal endometrium to simple hyperplasia to complex hyperplasia (complex hyperplasia without atypia/complex hyperplasia with atypia) in both MMR gene mutation carriers (groups I and II combined) and reference series (group III) as evident from Table 2. When comparing the combined group of MMR gene mutation carriers with the reference group, the average number of methylated loci was significantly higher in the former versus the latter group for normal endometrium (1.1 versus 0.3; $P = 0.0097$) and simple hyperplasia (2.0 versus 0.6; $P = 0.0016$), whereas no difference was seen for either type of complex hyperplasia (between groups or relative to each other). The fact that a hypermethylated state is present already in histologically normal endometrium may predispose MMR gene mutation carriers to early endometrial carcinoma development compared with sporadic cases.

Aberrant DNA methylation stratified the different histologic categories precisely in the same way as MMR defects in the Lynch syndrome group. For simplicity, we combined groups I, II, and III, and based on values given in Table 2, the average number of methylated loci was similar for normal endometrium versus simple hyperplasia (0.8 versus 1.0; $P = 0.5$), whereas normal endometrium was significantly less methylated relative to either complex hyperplasia without atypia (0.8 versus 2.7; $P < 0.0001$) or complex hyperplasia with atypia (0.8 versus 2.5; $P < 0.0001$). Additionally, in group I, methylation in endometrial carcinoma was similar to that in complex hyperplasia with atypia (2.1 in both).

Figure 2 shows promoter methylation frequencies for individual loci by histology, separately for the Lynch syndrome (groups I and II combined) and reference series. Examples of MS-MLPA tracings for normal endometrium, complex hyperplasia without atypia, and complex hyperplasia with atypia, all chosen from the reference series, are depicted in Fig. 3. Three loci, *CDH13*, *RASSF1(A)*, and *GSTP1*, were preferential targets for methylation in complex hyperplasia, whether originating from the Lynch syndrome group (75%, 63%, and 25%, respectively, for complex hyperplasia without atypia and 70%, 55%, and 30% for complex hyperplasia with atypia) or the reference group (85%, 69%, and 31% for complex hyperplasia without atypia and 62%, 54%, and 69% for complex hyperplasia with atypia). The same loci were frequently involved in endometrial carcinoma from group I (69%, 54%, and 15%). In contrast, lower methylation frequencies or no methylation at all were observed for these loci in simple hyperplasia and normal endometrium. Like MMR defects, aberrant tumor suppressor promoter methylation appeared before the diagnosis of endometrial carcinoma in group I (Table 3). For example, methylation at the *CDH13* promoter in normal endometrium and/or complex hyperplasia with atypia was observed 3 and 12 years before endometrial carcinoma in cases 2 and 5, respectively. Regarding interpretation of data given in Table 3, complex hyperplasia with atypia cells were occasionally interspersed with normal cells, making it impossible to separate "pure" areas of complex hyperplasia with atypia and normal cells for DNA extraction. In such cases, results from DNA-based assays were considered to represent complex hyperplasia with atypia but not normal tissue (whereas immunohistochemical evaluation always allowed distinction between complex hyperplasia with atypia and normal cells).

Discussion

Early detection is crucial for the outcome of malignant disease, and endometrial carcinoma constitutes no exception to the general rule (2). In our previous study, tumors from MMR gene mutation carriers who participated in surveillance were of an earlier stage than those diagnosed based on symptoms, and no deaths from endometrial carcinoma occurred in the surveillance group (13). Endometrial hyperplasia, which may precede endometrial carcinoma, is common: it may explain 15% of postmenopausal uterine bleeding in the general female population (23) and was present in 8% of MMR gene mutation carriers screened by Renkonen-Sinisalo et al. (13). It is therefore important to diagnose developing endometrial malignancies and their precursors as early as possible to guide management decisions, including the possible need of prophylactic hysterectomy.

To our knowledge, no systematic molecular genetic study of serial follow-up samples of endometrium from MMR gene mutation carriers has been conducted before. Some investigations do exist that report the presence of molecular alterations in hyperplasia from individuals with Lynch syndrome. Berends et al. (24) studied 15 women from Lynch syndrome families, who underwent endometrial sampling for nonmalignant reasons, and found MLH1 or MSH2 protein loss in 3 of 4 patients with endometrial hyperplasia (2 with complex hyperplasia with atypia and 1 with complex hyperplasia without atypia). de Leeuw et al. (25) analyzed five cases of hyperplasia from patients who also had endometrial carcinoma and observed absent MMR protein corresponding to the germ-line mutation in all cases of hyperplasia (three complex hyperplasia with atypia, one complex hyperplasia without atypia, and one simple atypical hyperplasia). Ichikawa et al. (26) describe a *MSH2* mutation carrier showing the loss of MSH2 protein in complex hyperplasia without atypia 7 months before endometrial carcinoma diagnosis. In a sporadic setting, Faquin et al. (27) conducted a retrospective study on patients who subsequently developed MSI(+) endometrial carcinoma, and in 1 of 13 patients, MSI was detectable in an endometrial sample with histologic diagnosis of "inactive" endometrium 7 years before the onset of endometrial carcinoma. Our study corroborates and extends these previous findings and shows that aberrant MMR protein expression, MSI, or tumor suppressor promoter methylation may be present in endometrial hyperplasia of various histologies, and even in normal endometrium, from MMR gene mutation carriers up to 12 years before endometrial carcinoma diagnosis (Table 3).

In colorectal carcinoma, it is debated if MSI analysis or immunostaining should be the primary method of prescreening for Lynch syndrome (28), whereas information available from endometrial carcinoma is limited. An important observation from our study was a higher sensitivity of immunostaining compared with MSI analysis in the detection of MMR defects in endometrial hyperplasia and endometrial carcinoma (Tables 2 and 3). Our data (with all hyperplasias originating from *MLH1* mutation carriers, except for one) are in agreement with de Leeuw et al. (25) who showed that all three cases of

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endometrial hyperplasia from *MLH1* mutation carriers were microsatellite-stable despite immunohistochemical loss of MLH1 expression. There may be a better concordance between immunohistochemical and MSI findings for *MSH2*-associated cases [ref. 25; a single complex hyperplasia without atypia from a *MSH2* mutation carrier in our study, displaying MSH2 protein loss and MSI with 3 of 3 markers (data not shown)]. Of note, even in the present Lynch syndrome series,

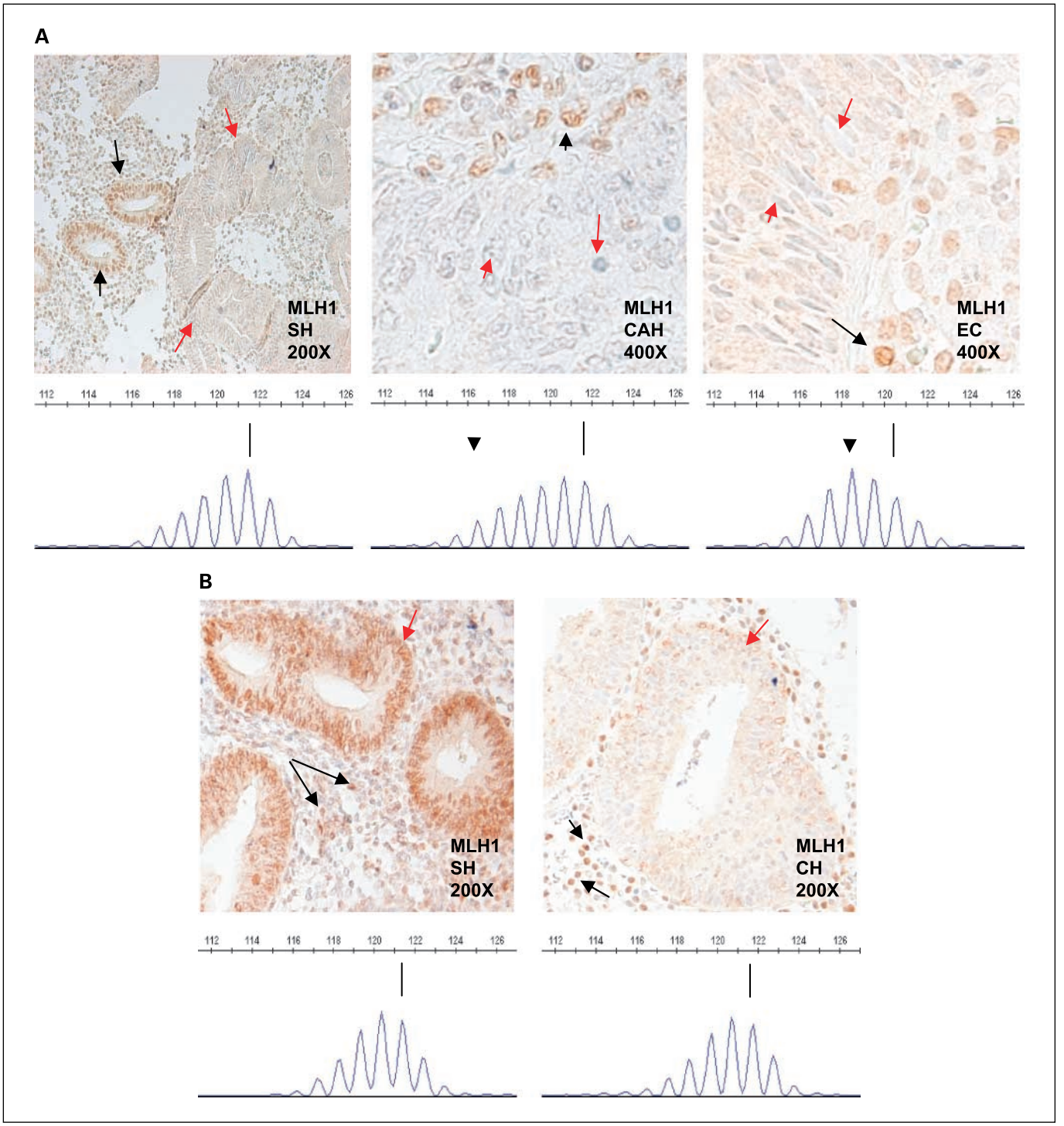

Fig. 1. Immunohistochemical images (with MLH1 antibody) and corresponding MSI plots (with *BAT25* marker) for patients 8 (*A*) and 24 (*B*) with germ-line mutation in *MLH1*. In patient 8, simple hyperplasia (*SH*; *top left*), complex hyperplasia with atypia (*CAH*; *middle*), and endometrial carcinoma (*EC*; *top right*) lacked MLH1 protein (*red arrows*), whereas stromal cells showed normal expression of MLH1 (*black arrows*) as indicated by brown staining. In the same patient, *BAT25* mononucleotide repeat was stable in simple hyperplasia and unstable in complex hyperplasia with atypia (*CH*) and endometrial carcinoma. *Short vertical lines,* constitutional peaks of 121 bp; *arrowheads,* size shifts indicative of MSI. In patient 24, simple hyperplasia expressed and complex hyperplasia without atypia did not express MLH1 protein, and both specimens were microsatellite-stable.

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one third of endometrial carcinoma tumors were stable with all three microsatellite markers (Table 2), and the proportion of stable tumors may be still higher for certain other types of tumors (brain and adenocarcinomas of the kidney) arising in MMR gene mutation carriers (20). There are several potential explanations for the absence of MSI despite immunohistochemical loss of MMR protein, one of them being intratumoral heterogeneity (20, 29).

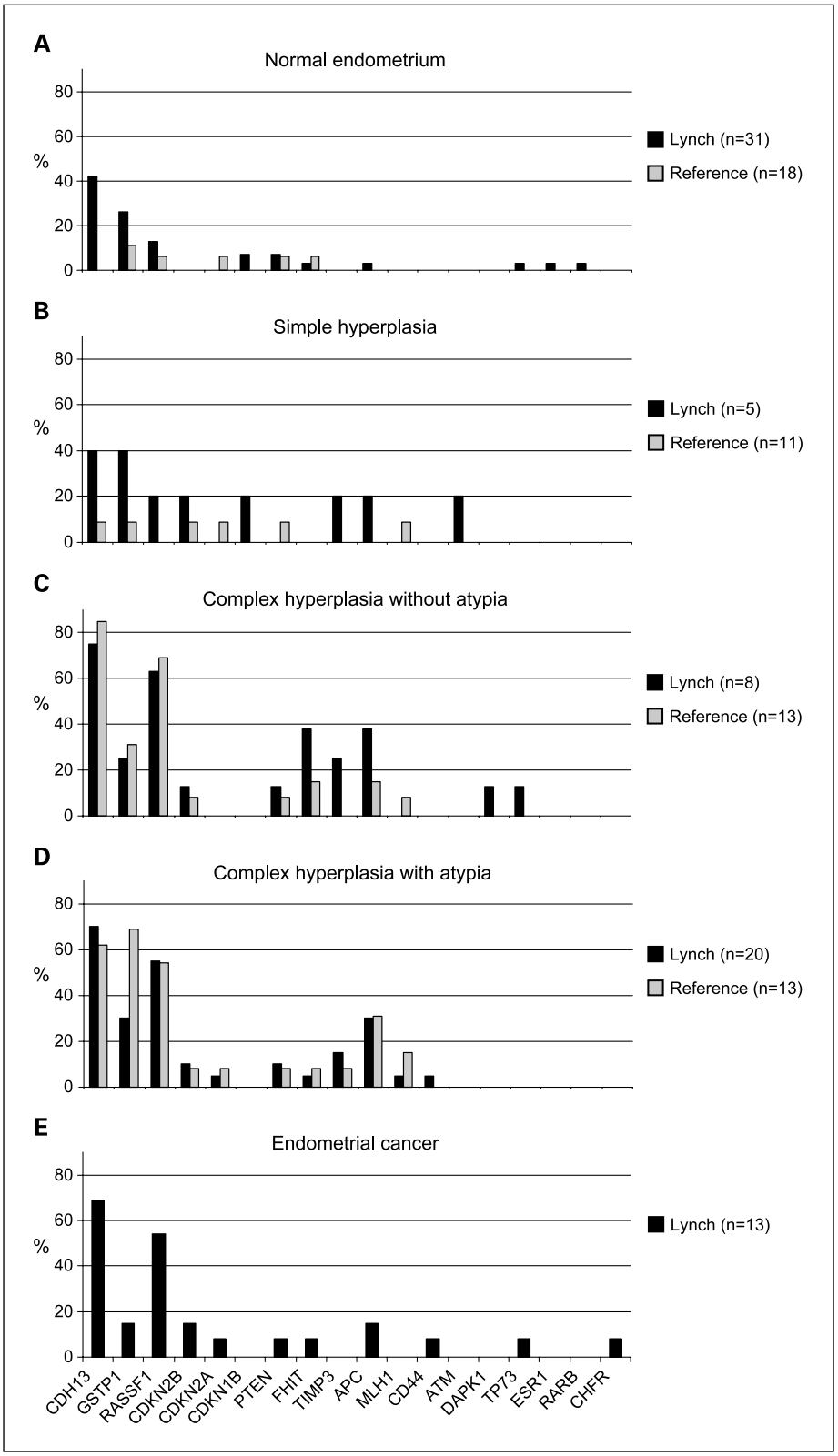

Fig. 2. Comparison of MS-MLPA results for individual tumor suppressor loci in the Lynch syndrome series (groups I and II combined) and reference series (group III). Loci that showed promoter methylation in at least one sample are included. Results for normal endometrium, proliferatory and secretory combined (*A*), simple hyperplasia (*B*), complex hyperplasia without atypia (*C*), complex hyperplasia with atypia (*D*), and endometrial carcinoma, group I (*E*).

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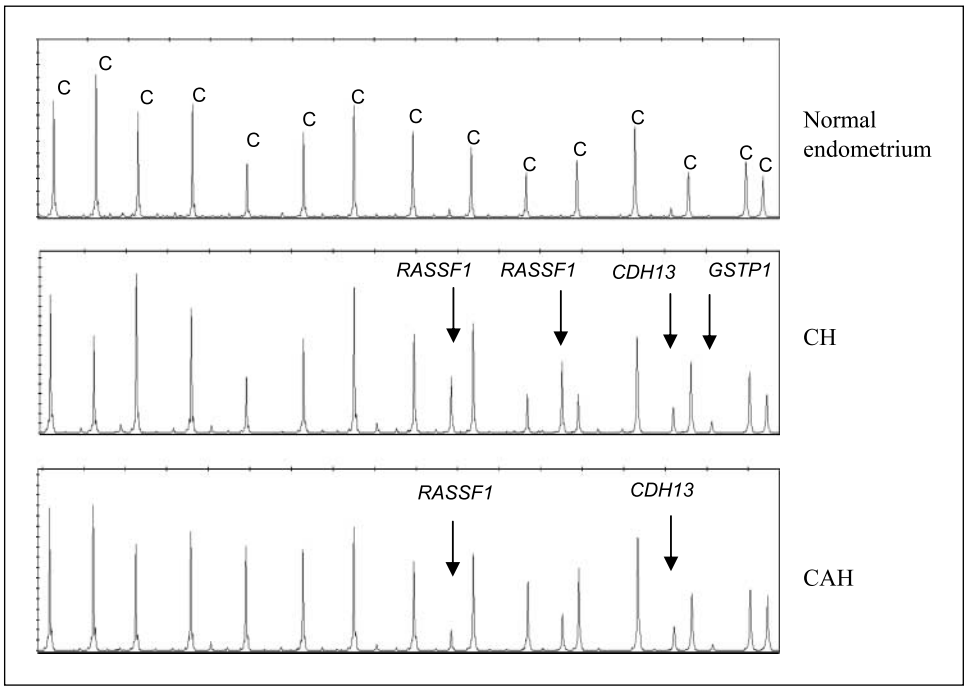

Fig. 3. Examples of MS-MLPA plots representing normal endometrium (*top*), complex hyperplasia without atypia (*middle*), and complex hyperplasia with atypia (*bottom*). Twenty-four different tumor suppressor loci were interrogated with specific probe pairs (two pairs of probes were included for *RASSF1*), combined with a set of control probes (*C*). The fragment size (increasing from left to right) identifies each locus. An amplification product (that is, visible peak) is obtained only if a given tumor suppressor locus is methylated (in contrast, control loci, which do not include *Hha*I sites, yield peaks invariably). *Arrows,* methylation of particular tumor suppressor gene loci in complex hyperplasia without atypia and complex hyperplasia with atypia, whereas no methylation is observed in normal endometrium.

Previous studies have implicated *PTEN* as an early marker of endometrial tumorigenesis, either through mutations or reduced expression, which may occur at premalignant stages (30, 31). Even more striking, PTEN-null glands may be present in histologically normal endometrium in 40% of premenopausal women, and because only a small minority of such individuals will ever develop endometrial carcinoma, it was suggested that the initial *PTEN* alterations are not rate-limiting (32). Although we did not study gene expression, presumable inactivation of *PTEN* by promoter methylation occurred in <15% of our endometrial samples (Fig. 2). By comparing PTEN expression in endometrial hyperplasia from individuals, who subsequently developed versus did not develop (sporadic) endometrial carcinoma, a recent investigation found that loss of PTEN expression was neither associated with nor a sensitive and specific marker of progression to endometrial carcinoma (33).

Our study emphasizes early and widespread tumor suppressor promoter methylation changes in endometrial tumorigenesis. In Lynch syndrome (groups I and II), increased DNA methylation, especially *CDH13* promoter methylation, was prevalent already in normal endometrium, being present in 42% of specimens (13 of 31) compared with 0% in the reference series (Fig. 2). Among 24 tumor suppressor genes studied, *CDH13*, *RASSF1*, and *GSTP1* were the most frequently methylated genes in endometrial hyperplasia and endometrial carcinoma (Fig. 2). The overall methylation profile in the present endometrial carcinomas diagnosed in association with a surveillance project was comparable with that in an independent series of 38 Lynch syndrome–related endometrial carcinomas examined previously (18). Furthermore, our data are broadly concordant with reports on sporadic endometrial carcinoma of the endometrioid type, whereas endometrial hyperplasias have not previously been characterized relative to DNA methylation changes to any significant extent. *CDH13*, which encodes H-cadherin, a calcium-dependent cell adhesion protein, has been reported to be methylated in 35% to 71% of endometrial carcinoma samples (34, 35). *RASSF1* codes for a Ras association domain family 1 protein (isoform A), a tumor suppressor, and is methylated in 33% to 85% of endometrial carcinoma (35–37) possibly correlating with advanced stage (38). *GSTP1* encodes glutathione *S*-transferase π, which is involved in carcinogen metabolism, and promoter methylation (present in 31%) may be associated with myometrial invasion of endometrial carcinoma (39). Additionally, MSI phenotype that occurs in 20% to 30% of sporadic endometrial carcinoma of the endometrioid type is mostly explained by biallelic *MLH1* promoter methylation (21, 22), whereas, not surprisingly, *MLH1* methylation was rare in the present series of endometrial carcinoma from Lynch syndrome (Fig. 2).

The literature on endometrioid carcinoma precursors is usually based on retrospective sample materials taken from symptomatic women. Heterogeneity of endometrial tissue, incomplete representativeness of the sampled material, poor reproducibility of histopathologic diagnostics, and limited possibility to monitor the clinical behavior, particularly of complex hyperplasia without atypia and complex hyperplasia with atypia, have all hampered studies on the evolution of the endometrioid neoplastic process. Also, it is uncertain to which extent the diagnostic procedure and nonsurgical therapeutic intervention may affect the natural history of the neoplastic process. Largely based on the seminal study by Kurman et al. (4), nuclear atypia has been considered as the most important risk factor for progression to malignancy. In agreement, in sporadic cases investigated by Esteller et al. (40), *MLH1* promoter methylation was primarily restricted to complex hyperplasia with atypia, the difference in methylation frequencies between complex hyperplasia without atypia and complex hyperplasia with atypia being statistically significant [1 of 33 (3%) versus 7 of 21 (33%); $P = 0.0039$]. In the

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present study on Lynch syndrome and sporadic cases, aberrant tumor suppressor promoter methylation, MMR protein expression, and MSI were equally common in complex hyperplasia without atypia versus complex hyperplasia with atypia, and the alteration frequencies in these complex hyperplasias were comparable with endometrial carcinoma but different from simple hyperplasia and normal endometrium. Our Lynch syndrome series mainly consisted of *MLH1* mutation carriers and it remains to be seen if tumorigenesis is similar or different in carriers of mutations in other MMR genes, such as *MSH6*, which is particularly associated with endometrial cancer predisposition (41). Although confirmation in larger series is warranted, our findings suggest that complex hyperplasia without atypia and complex hyperplasia with atypia are equally important as precursor lesions of malignancy. The present results are supported by Pijnenborg et al. (37) who observed *RASSF1A* promoter methylation in 4 of 8 (50%) among endometrial hyperplasia specimens, and although the exact histology was not specified, the authors wrote that "atypia was present in only one case with endometrial hyperplasia, indicating that *RASSF1A* methylation was not restricted to those cases with cellular atypia."

In conclusion, our results provide important new insights into the timing and molecular nature of the critical events involved in endometrial tumorigenesis and may turn out useful in the design of improved surveillance strategies for endometrial carcinoma in the future. Molecular alterations in biopsy specimens could serve as an indicator for prophylactic hysterectomy, for example, in MMR mutation carriers who would not primarily opt for prophylactic surgery (42).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Clinical Cancer Research

Molecular Analysis of Endometrial Tumorigenesis: Importance of Complex Hyperplasia Regardless of Atypia

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