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Genes Involved in DNA Repair Are Mutational Targets in Endometrial Cancers with Microsatellite Instability[1]

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ABSTRACT

Microsatellite instability (MSI) is observed in a subset of endometrial cancers (ECs) and is attributed to defects in mismatch repair. Mismatch repair deficiency allows for accumulation of mutations in the coding repeats of key target genes, which may be involved in the initiation and progression of MSI+ EC. We examined genes implicated in DNA repair pathways in 38 MSI-high (MSI-H), 10 MSI-low, 25 microsatellite stable ECs, and a selected panel of associated premalignant hyperplasias. Genetic alterations were correlated to histopathological data, including tumor grade and stage. Somatic frameshift mutations were observed in *hMLH3, hMSH3, hMSH6, CHK1,* and *BAX* genes in MSI-H endometrial hyperplasias and cancers, whereas mutations in *ATR* and *CDC25C* were observed only in MSI-H ECs. Increased mutation frequency in DNA damage response pathway genes including *ATR, CHK1,* and *BAX* demonstrated a significant trend with advancing tumor grade ($P < 0.05$). Our observations of the same mutations at short coding mononucleotide repeats in both premalignant lesions and tumors and association of increased frequency of mutation accumulation with advancing tumor grade suggest that these alterations may play a role in the development and progression of MSI+ EC.

INTRODUCTION

Endometrial adenocarcinoma arises from the uterine lining through a continuum of distinct morphological stages characterized as simple and complex hyperplasia with or without atypia (1, 2). About 20% of ECs[3] occur due to defects in MMR, and these tumors are characterized by accumulation of alterations at microsatellite loci, known as MSI or mutator phenotype (3, 4). MMR-deficient tumor cells are susceptible to progressive accumulation of somatic mutations at repeated sequences in the coding regions of key target genes, such as DNA repair. A model has been proposed for the tumor mutator pathway, in which the first step is the functional inactivation of *hMLH1* or *hMSH2* (primary mutators), followed by the inactivation of other secondary mutator genes (*hMLH3*, *hMSH3*, and *hMSH6*) by frameshift mutations in their coding repeats induced by the primary mutators (5). As a consequence, the MMR system is further impaired, allowing for faster accumulation of genetic instability and progression to neoplasia.

MMR-deficient cells may also accumulate mutations in genes that are involved in key cellular pathways, further acquiring traits that are advantageous in the tumorigenic process. In this regard, genes in the DNA damage response pathway are good mutational candidates because of their importance in cell cycle regulation. A disruption in cell cycle regulation leads to the propagation of defective DNA and increased cellular proliferation, traits characteristic of cancer cells. For example, the evolutionarily conserved kinase ATR is activated in response to DNA damage and phosphorylates CHK1 and CHK2 (6–8). Phosphorylation of CDC25C by CHK1 inhibits CDK1, resulting in G_2 arrest and mitosis prevention. Because *ATR*, *CHK1*, and *CDC25C* function to maintain genomic integrity by preventing mitosis in response to DNA damage and also contain mononucleotide repeats in their coding regions, they are potential mutation targets in MSI+ cancer cells. ATR also phosphorylates and activates p53, BRCA1, and BRCA2 (8), which are involved in cell cycle progression, centrosome duplication, DNA damage repair, cell growth, apoptosis, transcriptional activation, and repression (9). *BRCA1* and *BRCA2* contain coding mononucleotide repeats and may be susceptible to incur alterations in MMR-deficient cells.

Several other genes are also involved in maintaining the integrity and stability of the genome (10). DNA helicases unwind double-stranded DNA and play a role in DNA replication, recombination, and repair (11). *BLM*, *WRN*, and *RECQL* are evolutionarily conserved members of the RecQ helicase family (10, 12, 13), and alterations in these genes result in a strong predisposition to cancer. *BLM*, *RECQL*, and *WRN* contain coding mononucleotide repeats, and mutational inactivation of these genes in MMR-deficient cells may play a role in MSI+ tumorigenesis by possibly reducing defective DNA repair (12).

Functional inactivation of many of these genes has been examined in MSI+ cancers, mainly colorectal cancers (3, 14, 15); however, their specific contributions to MMR-deficient EC and, in particular, premalignant uterine hyperplasias have not been systematically examined. In this study, we investigated candidate genes implicated in DNA repair pathways as possible targets of somatic mutations in MSI+ ECs and in adjacent premalignant hyperplasias to assess the contribution of such mutations to the initiation and progression of EC. We also examined the correlation between mutations in these pathways with respect to tumor histopathology. Our results suggest a contributory role of somatic mutations in DNA repair genes in MSI+ endometrial tumorigenesis and progression.

MATERIALS AND METHODS

Patient Population. Paraffin-embedded endometrial adenocarcinomas were collected from young (<50 years of age) patients diagnosed during a 5-year period (1989–1993) using a provincial database (Ontario Cancer Registry; Ref. 4). Among the cases identified, 48 were MSI+. Specific data such as patient survival, tumor grade and stage, and recurrence of EC and other cancers were collected (up to 10-year follow-up). All protocols, including specimen accrual, were approved by the Ethics Committee of the University of Toronto.

Histopathological Assessment. Samples consisting of primary endometrial adenocarcinomas were cut into 5-μm sections, stained with H&E, and classified according to International Federation of Gynecologists and Obstetricians (FIGO) criteria (2). Grading and staging of tumors as well as the classification and extent of associated hyperplasias were assessed by W. C. (a pathologist with expertise in gynecologic pathology). Areas enriched (>70% cellularity) in normal, hyperplastic, and tumor cell populations were identified and marked for microdissection from 10-μm unstained sections; the samples were used for DNA extraction, and a final 5-μm H&E-stained section was used

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[3] The abbreviations used are: EC, endometrial cancer; MSI, microsatellite instability; MMR, mismatch repair; MSI-H, MSI-high; MSS, microsatellite stable; SH, simple hyperplasia; AH, atypical hyperplasia.

Table 1 *Primers spanning the coding mononucleotide repeats of the candidate DNA repair genes are listed below, together with the corresponding bp size of the PCR amplicon*

PCR conditions are as follows: initial denaturation for 95°C for 2 min, followed by 35 cycles of amplification [95°C for 30 s, annealing temperature (°C) for 30 s, 72°C for 30 s, and, finally, 72°C for 5 min, 4°C cooling bath].

Gene	Repeat	Forward primer	Reverse primer	Product size (bp)	T_m (°C)	$MgCl_2$ (mM)
hMSH3	(A)8	5′-CTATCTTCTGTGCATCTCTG-3′	5′-ACAATGCCAATAAAAATGTTGC-3′	75	58	2.5
hMSH6	(C)8	5′-TGATGGTCCTATGTGTCGC-3′	5′-GTCTTCGTAATGCAAGGATG-3′	96	58	1.5
hMLH3	(A)9	5′-ACATAGTGCTCAGACAGAG-3′	5′-CTTCTTCCACAATTGCTAGA-3′	57	56	3.5
	(A)8	5′-TAAGTAAAGAATCTGGTCAAT-3′	5′-GCTGTAGGTTCATTCTCTA-3′	82	54	3.0
ATR	(A)10	5′-CTTCTGTCTGCAAGGCCATT-3′	5′-AGCAAGTTTTACTGGACTAGG-3′	65	60	1.0
CHK1	(A)9	5′-CTGTCAGGAGTATTCTGACT-3′	5′-GAGCTAGAGGAGCAGAATC-3′	80	58	2.0
CHK2	(T)6	5′-CTTAGCCTTGCATCATCAAG-3′	5′-AAGTTTACTACTTACAATTCCAA-3′	82	58	3.5
CDC25C	(A)8	5′-TCAAGGACAACACAATACCAG-3′	5′-CTGAGCTTTCCTTGGCCAG-3′	64	60	3.5
CDK1	(A)7	5′-CTACAGGTCAAGTGGTAGC-3′	5′-CGAATTGCAGTACTAGGAAC-3′	76	58	2.5
BAX	(G)8	5′-ATCCAGGATCGAGCAGGGCG-3′	5′-ACTCGCTCAGCTTCTTGGTG-3′	94	64	1.5
BRCA1	(A)8	5′-GTTGTTCTAGCAGTGAAGAG-3′	5′-CTGCTGTGCCTGACTGGC-3′	64	60	2.0
BRCA2	(A)8	5′-CTAATCTGTTATTTTGGTAG-3′	5′-ATCTATGCAAAGTACAAGG-3′	116	52	2.5
	(A)8	5′-TATTTATGCTATACATGARG-3′	5′-AAACTGGGCTGAACAGT-3′	91	50	2.0
	(A)8	5′-TGAAAGAGCTAACATACAG-3′	5′-CGGTAGTTGTTGATACTG-3′	58	52	2.0
BLM	(A)9	5′-GTAAGTAGCAACTGGGCTG-3′	5′-CAGCAGTGCTTGTGAGAAC-3′	88	58	1.5
RECQL	(A)9	5′-TACGGAAAGGCAACAAGAG-3′	5′-CGGCATCAGAATCCTCTAA-3′	83	56	2.0
WRN	(A)8	5′-TGGACTCTGCAAATAGGACA-3′	5′-CTGCTGTGCAGTTGTTTCC-3′	64	58	2.5

for the confirmation of proper alignment and histopathology with preceding sections.

MSI Analysis. DNA was extracted from each cell population (normal, hyperplastic, and tumor) per previously described protocols (4) and analyzed for MSI using the National Cancer Institute-recommended panel of five microsatellite markers for normal and tumor tissue and BAT-25 and BAT-26 markers for hyperplasias (4, 16). MSI was defined by the presence of altered and/or additional alleles in the PCR-amplified product of tumor or premalignant DNA as compared with matched normal DNA. Tumors were classified as MSI+ if alterations were observed in at least 40% of the analyzed marker loci. Hyperplasias were designated as MSI+ if one of two or both analyzed loci showed instability.

Analysis of Candidate Genes. Forty-eight MSI+ (38 MSI-H, ≥40% MSI; 10 MSI-low, <40% MSI) and 25 MSS endometrial adenocarcinomas were examined for somatic mutations in coding mononucleotide repeats of genes involved in DNA repair pathways including MMR, DNA damage response, and nucleotide excision repair. Sequences of candidate genes were obtained from GenBank,[4] and primers were designed that span the coding mononucleotide repeat stretches, producing PCR amplicons of 57–116 bp. One primer of each pair was end-labeled with [γ-^{33}P]ATP, and specimens were analyzed for alterations in candidate genes by PCR and gel electrophoresed as described previously (4). Primers and amplification conditions are described in Table 1. Putative mutations were confirmed by manual DNA sequencing. For ECs harboring somatic mutations, associated hypeplasias were analyzed for the presence or absence of the same genetic alterations.

Statistical Analysis. Association of mutation status with tumor grade and stage was examined by considering all DNA repair pathways together and by specifically analyzing each DNA repair pathway: MMR; DNA damage response; and nucleotide excision repair. Contingency tables were constructed, and tests for association were performed using a two-sided Fisher's exact test. Due to the small sample size, tumor stage was analyzed as a binary variable (stage 1 *versus* stage 2), and no survival analysis was carried out. No analysis was performed on mutation frequencies in the nucleotide excision repair pathway because we found only a single genetic alteration.

Possible trends in the probabilities associated with the presence of mutations were tested with respect to tumor grade using Bartholomew's test (17). The test is based on the following statistical formula:

$$\bar{\chi}^2 = \frac{1}{pq}\sum_{i=1}^{m} n_i(p_i - p)^2$$

where p_i is the proportion of mutations in each tumor grade i, $p(= 1 - q)$ is the overall proportion and the sum overall tumor grades ($m = 3$), and n_i is the number of individuals in each tumor grade.

[4] www.ncbi.nlm.nih.gov.

RESULTS

MSI Analysis of Hyperplasias. Ten of 14 MSI-H tumors had adjacent hyperplasia. Three cases had SH, five had AH, and two cases had both SH and AH. Eleven of 12 hyperplasias displayed MSI in at least one of the loci analyzed (BAT-25 and BAT-26), and seven of these hyperplasias displayed instability at both loci (Fig. 1*A*). Furthermore, the instability profile, as determined by the type and/or extent of alterations at the microsatellite loci, was identical among hyperplasias and associated tumors, which indicated a progressive accumulation of genetic instability and was consistent with clonal evolution of SH and AH to endometrioid adenocarcinoma (Fig. 1*B*).

A

Case ID	MSI Markers	
	BAT-25	BAT-26
8SH	-	+
8T	+	+
15SH	+	+
15T	+	+
59SH	+	+
59AH	+	+
59T	+	+
62AH	-	+
62T	+	+
87AH	+	+
87T	+	+
98SH	-	-
98T	+	+
101AH	+	+
101T	+	+
110SH	+	-
110AH	+	-
110T	+	+
124AH	+	+
124T	+	+
184AH	+	+
184T	+	+

B

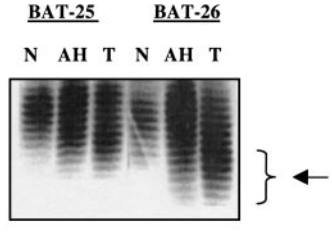

Fig. 1. *A*, MSI analysis of endometrial tumors with associated hyperplasias using BAT-25 and BAT-26 markers. +, presence of instability; −, absence of instability; *T*, tumor. *B*, representative panel illustrating the progressive accumulation of MSI using BAT-25 and BAT-26 markers in normal endometrium (*N*), hyperplasia (*AH*), and tumor (*T*) for case 124. *Arrow* indicates extent of MSI.

A

CASE ID	GENES: Mismatch Repair				DNA Damage Response				NT Repair	STAGE	SURVIVAL
	hMLH3 (9)	*hMLH3 (8)*	*hMSH6*	*hMSH3*	*ATR*	*CHK1*	*CDC25C*	*BAX*	*BLM*		
8SH						delA					
8T					delA	delA				1A	A
15SH	delA							delG			
15T	delA					delA		delG		1B	D
59SH											
59AH											
59T					insA	delA	delA			1B	D
62AH											
62T								delG		1B	A
65T									delA	1B	D
87AH											
87T								delG		1B	A
98SH											
98T								delG		1C	A
101AH	delA										
101T	delA		delA							1A	A
110SH						insA					
110AH						insA					
110T						insA				1A	A
114T								delG		1B	A
124AH	delA		delA		delA						
124T	delA		delA		del2A					1B	A
150T					delA			delG		2B	A
179T				delC						1A	A
184AH				insC							
184T				insC						1B	A

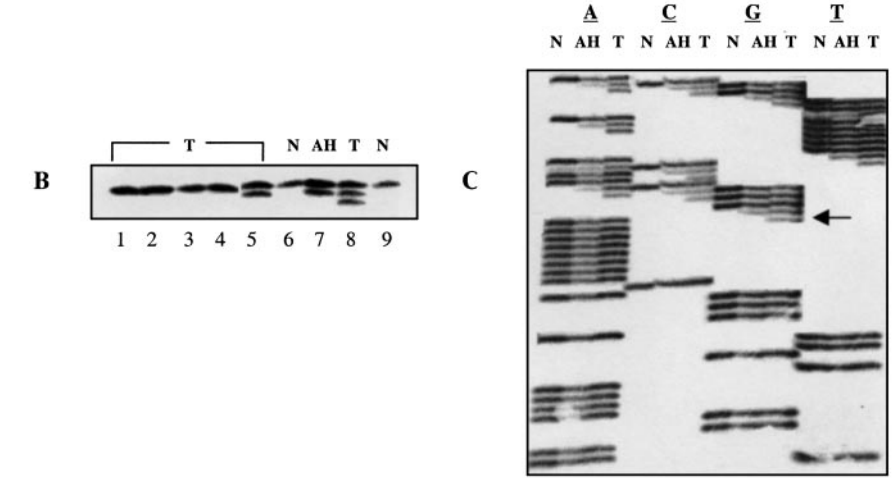

Fig. 2. *A*, mutations found in MSI-H ECs ($n = 14$) and associated hyperplasias ($n = 10$) in coding repeats of examined genes. *T*, tumor; *A*, alive; *D*, deceased. *B*, example of somatic mutations found in the (A)8 coding repeat of the *hMSH3* gene in AH and tumor; *Lanes 1–4* represent tumors without somatic mutations in the coding repeat of the *hMSH3* gene; *Lane 5* represents a tumor harboring a deletion of A in the coding repeat; *Lanes 6–8* represent normal endometrium, AH with A deletion, and tumor containing AA deletion, respectively. *Lane 9* represents normal endometrium (*N*) *C*, sequence analysis of mutations represented in *Lanes 7* and *8* in *B*. Frameshift mutations caused by deletion of 1 bp (*AH*) and 2 bp (*T*) for case 124 are shown. *Arrow* indicates frameshift in subsequent sequence caused by A deletions.

Somatic Mutations in Candidate Genes in MSI+ EC and Hyperplasias. Fourteen of 38 MSI-H tumors and 6 of 10 associated hyperplasias displayed somatic alterations in the coding mononucleotide repeats in at least one of the candidate genes examined (Fig. 2*A*); none of the MSI-low tumors showed somatic mutations in the examined genes. A representative panel of MSS tumors ($n = 25$), matched for tumor grade and stage with MSI-H EC, was also screened and revealed no somatic alterations in the examined coding mononucleotide repeats.

We detected 24% (9 of 38) of somatic mutations in the coding repeats of MMR genes including *hMLH3*, *hMSH3*, and *hMSH6* in MSI-H EC and in 7 of 9 adjacent premalignant hyperplastic lesions. Mutations of target genes in the DNA damage response pathway (*ATR*, *CHK1*, *CDC25C*, and *BAX*) were observed in MSI-H EC, and among these, *CHK1* and *BAX* mutations were also observed in associated hyperplasias. No alterations were detected in the coding repeats of *CHK2*, *BRCA1*, and *BRCA2*, possibly suggesting that these specific repeats may not be susceptible to secondary mutations in MSI+ EC. Similarly, genes from the nucleotide excision repair pathway did not accumulate somatic mutations in their coding repeats in MSI+ EC, with the exception of one *BLM* mutation found in a single tumor (Fig. 2*A*).

Mutation Distribution in MSI+ EC and Statistical Analysis. A significant association of grade and stage of EC with either overall mutation status or mutations in each pathway (Table 2) was not observed by Fisher's exact test, which is not surprising, given the limitations of our small sample size. However, the test for an increase in proportions (Bartholomew's test) was significant ($P < 0.05$) in determining a trend with advancing tumor grade (Table 2C) in the DNA damage response pathway.

DISCUSSION

MMR-deficient tumors have an increased tendency to accumulate mutations in genes with coding repeats; however, there seems to be a selective pressure for certain genes to be mutated, making them preferential targets and potential contributors to the initiation and/or progression of MSI+ EC. From the examined panel of DNA repair genes, alterations were observed in genes involved in MMR (*hMSH3*, *hMSH6*, and *hMLH3*), DNA damage response (*ATR*, *CHK1*, *CDC25C*, and *BAX*), and nucleotide excision repair (*BLM*), and a significant proportion (6 of 10) of endometrial hyperplasias also harbored the same mutations. Coding region repeats in these genes either precede or occur in important functional domains. Frameshift mutations, therefore, are predicted to result in proteins lacking critical domains. Functional inactivation of many of these genes is already implicated in cancer predisposition and development (5–14, 18–20), and somatic mutations in their coding repeats have been reported previously in MSI+ colorectal and gastric cancers (3, 14, 15, 19, 20). To date, there are no known estimates of the overall mutation status

Table 2 *Association of somatic mutations found in DNA repair pathways with tumor grade and stage: A, all pathways combined (MMR, DNA damage response, and nucleotide excision repair); B, MMR, and C, DNA damage response*

A significant trend was observed between grade and mutation status by the use of Bartholomew's test ($P < 0.05$) in the DNA damage response pathway. No significant associations were found between tumor grade and stage with respect to mutations status by Fisher's exact test. +, mutation positive; −, mutation negative.

	DNA repair				
	Total	+	−	Mutations (%)	
A. DNA repair					
Grade					
1	21	7	14	33	
2	9	4	5	44	
3	8	5	3	63	
Stage					
1	35	14	11	40	
2	3	2	1	67	
Total	38	16	22	42	
B. MMR					
Grade					
1	21	3	18	14	
2	9	2	7	22	
3	8	1	7	13	
Stage					
1	35	5	30	14	
2	3	1	2	33	
Total	38	6	32	16	
C. DNA damage response					
Grade					
1	21	3	18	14	$(P < 0.05)^a$
2	9	2	7	22	
3	8	4	4	50	
Stage					
1	35	8	27	23	
2	3	1	2	33	
Total	38	9	29	24	

[a] A significant trend was observed between grade and mutation by Bartholomew's test in the DNA damage response pathway.

of these genes in MSS EC, and similarly, we did not observe mutations of these genes in our series of MSS tumors. These observations suggest that certain genes are more susceptible to alterations in MSI+ rather than MSS EC, underlying the distinct molecular genetic mechanisms between MSI and MSS tumorigenesis. Our results suggest that certain alterations in a genetic pathway, if growth advantageous for specific tissues, are selected for in tumor cells, allowing for clonal expansion. If such alterations are observed in associated premalignant lesions, then it is likely that they aid in the progression to carcinogenesis.

Somatic mutations in *hMSH3* and *hMSH6* have been studied extensively in MSI+ colorectal, endometrial, and gastric cancer; however, a role for *hMLH3* has only recently emerged in the context of MSI+ colorectal cancers (19). *hMLH3* contains two coding mononucleotide repeats, and we found both of them to be mutated in EC. The (A)9 repeat had a higher mutational frequency than the (A)8 repeat of *hMLH3* in both tumor and associated hyperplasia (Fig. 2*A*), suggesting that it may be preferentially targeted, followed by the (A)8 repeat. Furthermore, it is also possible that the multiple alterations in *hMLH3* observed in the same tumor (Fig. 2*A*, cases 101 and 124) are biallelic. Our study is the first to address the role of *hMLH3* alterations in MSI+ EC and to further implicate somatic mutations of *hMLH3*, *hMSH3*, and *hMSH6* genes in the early stages of MSI+ endometrial tumorigenesis by demonstrating their occurrence in associated hyperplasias adjacent to tumors harboring the mutations.

Response to DNA damage is crucial in the maintenance of genomic stability and cellular integrity. Our examination of genes in this pathway implicates *CHK1* and *BAX* as early mutational targets in the development of EC because premalignant lesions harbored somatic mutations that were maintained in tumors. Somatic mutations of *ATR* and *CDC25C* were observed in tumors only, implicating them as mutational targets involved in the later stages of MSI+ EC in the same pathway.

Because MSI+ tumors display a high background of genetic instability, it is difficult to establish which alterations are likely to play a key role in carcinogenesis. Five criteria have recently been proposed to establish whether mutations occur preferentially in certain genes, and whether they are likely to contribute to MSI+ tumorigenesis (14). These criteria are as follows: (*a*) high mutational frequency; (*b*) biallelic inactivation; (*c*) role for the candidate target gene in a growth suppressor pathway; (*d*) occurrence of alterations within the same genes and/or same pathway in MSS tumors; and (*e*) in *vitro* or in *vivo* functional studies to support a role of mutations in functional inactivation of candidate genes. Based on our study, we propose the inclusion of one more criteria, the occurrence of somatic mutations in premalignant lesions, such as endometrial hyperplasias or colonic adenomas, in the same target genes. The progressive nature of genetic instability accumulation was clearly illustrated in one case by the presence of a single-base deletion in the coding (A)8 repeat of the *hMSH3* gene in the hyperplasia, whereas the tumor contained a 2-bp deletion at the same site (Fig. 1*B*).

Our findings agree with the haploinsufficiency model of the mutator phenotype that has been recently proposed by Yamamoto *et al.* (15). According to this model, monoallelic mutations in several target genes that belong to the same pathway accumulate in cancer cells during tumor progression and inactivate the pathway. Six of 14 tumors (42%) accumulated somatic mutations in multiple genes in MMR and DNA damage response pathways. Because maintenance of genome stability depends on the cell's ability to repair and properly respond to DNA damage, accumulation of heterozygous mutations in multiple genes whose products have synergistic roles at different points in a specific pathway reduces the homeostatic threshold amount of the corresponding proteins in the pathway.

We observed a significant relationship between mutation status and tumor grade only in the DNA damage response pathway. Given the progressive nature of mutation accumulation at coding region repeats of key target genes in MSI+ tumors, we expected to see that the more advanced tumors (higher grades) were associated with a higher number of such alterations. However, when the overall mutation status was assessed for such an association, we did not observe a significant increase in the number of mutations with advancing tumor grade and stage. Similarly, we did not observe an increased number of mutations in MMR genes with advancing tumor grade and stage. This does not indicate that these alterations are not important because mutations in MMR genes may play a role in the initiation rather than progression of MSI+ EC. It makes sense to have MMR genes mutated early because they can contribute to further genetic instability at microsatellites of regulatory genes, resulting in their inactivation and thereby providing cells with a growth advantage.

In summary, genes involved in MMR and DNA damage response pathways are important for the maintenance of the normal endometrium, and inactivation of these genes may contribute to the initiation and progression of EC with MSI.

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