Methylation of *Adenomatous Polyposis Coli* in Endometrial Cancer Occurs More Frequently in Tumors with Microsatellite Instability Phenotype

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Abstract

Differential methylation is an important epigenetic control mechanism, which has been implicated in the development of a variety of cancers. Methylation of promoter regions of normally unmethylated tumor suppressor genes leads to transcriptional inactivation and ultimately to tumor formation. We hypothesized that epigenetic inactivation of *adenomatous polyposis coli* (*APC*), a key player in the suppressor pathway, may contribute to the development of endometrial cancer. We investigated *APC* methylation in endometrial adenocarcinoma specimens obtained from a series of patients (*n* = 114) and compared methylation profiles with microsatellite instability (MSI+) status. DNA microdissected from formalin-fixed, paraffin-embedded matched normal and tumor specimens, and a subset of associated endometrial hyperplasia was subjected to methylation-specific PCR of the *APC* promoter 1A region. Tumor-specific hypermethylation of *APC* with corresponding unmethylated normal endometrial tissue occurred in 43% (17 of 40) of MSI+ cases (*P* = 0.0007) and 16% (12 of 74) of microsatellite stable cases (*P* = 0.04). Interestingly, tumor tissue was unmethylated with normal tissue displaying *APC* methylation in 4% (5 of 114, 2 MSI+ and 3 microsatellite stable) of cases. Endometrial cell lines AN3CA, RL95–2, and HEC-1B all displayed exclusive methylation of promoter 1A, and treatment of the AN3CA cell line with the demethylating agent 5-aza-2′-deoxycytidine exhibited re-expression of *APC* as confirmed by RT-PCR analysis. Our results demonstrate *APC* methylation in endometrial cancer for the first time and show that *APC* hypermethylation occurs at an increased frequency in MSI+ endometrial tumors (*P* = 0.01).

Introduction

The Wnt cell-signaling pathway is among the key cellular developmental pathways. Disregulation of this pathway has been implicated in the initiation and progression of cancer. *APC* is an important tumor suppressor gene of the Wnt signaling pathway. *APC* is located on chromosome 5q21 and consists of 8535 bp of coding sequence spanning a total of 21 exons (1). Two promoters, 1A and 1B, have been identified for *APC* (2). Promoter 1A is shown to regulate transcription of the most common isoform of *APC*, which is encoded by exons 1–15. Mutations of *APC* predominantly occur in both inherited and sporadic colorectal cancers. Somatic *APC* mutations have been described in a number of extracolonic cancers, including gastric, pancreatic, thyroid, and ovarian (3) but rarely, if any, in ECs (4).

The *APC* protein carries diverse functions, including regulation of β-catenin, a key component of the Wnt signaling pathway. In the absence of growth and differentiation signals, β-catenin forms a complex with APC, axin, and glycogen synthase kinase-3β. APC regulates β-catenin stability and turnover by mediating β-catenin degradation by the ubiquitin-proteasome system. Either mutation of β-catenin or loss of APC function results in the stabilization and subsequent nuclear accumulation of β-catenin, which leads to the activation of several downstream target genes such as c-myc, *cyclin D1*, and *matrilysin* (5). β-catenin mutations occur in ECs and are described more frequently in MSS tumors (6, 7). Although the majority of endometrial tumors are MSS, a subset (−20%) is recognized by the MSI+ phenotype resulting from mismatch repair deficiency (8). Occurrence of β-catenin mutations implicates the Wnt signaling pathway in endometrial tumorigenesis, however, these mutations are seen at a low frequency, suggesting that alternative mechanisms may also contribute to the dysregulation of the Wnt pathway. One possible mechanism is functional inactivation of *APC* caused by mutations, allelic deletions, or via promoter hypermethylation.

Methylation of normally unmethylated genes, particularly tumor suppressors, has been shown to result in gene silencing and may ultimately lead to the development of cancer. Although there has been no evidence of *APC* promoter 1B methylation, methylation of promoter 1A and the accompanying loss of the *APC* transcript have been shown to occur in colorectal (9), breast, lung (10), esophageal, and pancreatic cancers (11). In this study, we investigated whether inactivation of *APC* via promoter hypermethylation contributes to the development of EC. We report that *APC* hypermethylation is significantly associated with the MSI+ phenotype in endometrial tumors.

Materials and Methods

Cell Lines. EC cell lines AN3CA, HEC-1B, RL95–2, KLE, and colon cell line HT-29 were obtained from the American Type Culture Collection (Manassas, VA) and were maintained as described previously (12), with the exception of KLE, which was maintained in a 1:1 mixture of Ham’s F-12 and DMEM supplemented with 10% FBS (Invitrogen Corp., Carlsbad, CA). DNA extracted from cells using the DNeasy Tissue Kit (Qiagen, Inc., Mississauga, Ontario, Canada).

Clinical Samples. Formalin-fixed, paraffin-embedded tissue sections of endometrial adenocarcinomas, as well as their associated hyperplasias and matched normal specimens (*n* = 114), were obtained from patients diagnosed at ≤50 years of age, according to a protocol approved by the Human Ethics Committee, University of Toronto. Five-μm H&E-stained sections were examined by a pathologist (W. C.) with expertise in gynecological oncology for confirmation of histology and cellularity. To minimize the field effect of adenomatous polyposis coli (APC), a subset of associated endometrial hyperplasia was subjected to methylation-specific PCR of the *APC* promoter 1A region in accordance with *APC* promoter hypermethylation contributes to the development of EC. We report that *APC* hypermethylation is significantly associated with the MSI+ phenotype in endometrial tumors.

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SssI methyltransferase (New England Biolabs, Beverly, MA) and subsequently described previously (11). Normal lymphocyte DNA supermethylated with sodium metabisulfite alone was included as an unmethylated control. Untreated normal lymphocyte DNA was also included as a negative control.

**5′-TGTGAGGGTATATTCTGGAGGAT-3′** and **APC-1A-UR, 5′-TGTGAGGGTATATTCTGGAGGAT-3′** and **APC-1A-MR, 5′-TGTGAGGGTATATTCTGGAGGAT-3′**. This was amplified by 90-bp product (nucleotides 635–724). PCR was carried out at an annealing temperature of 65°C (unmethylated primers) or 57°C (methylated primers).

For promoter 1B, the unmethylated and methylated primers were designed as described previously (11). Normal lymphocyte DNA supermethylated with SssI methyltransferase (New England Biolabs, Beverly, MA) and subsequently treated with sodium metabisulfite served as the methylated control. Normal lymphocyte DNA treated with sodium metabisulfite alone was included as an unmethylated control. Untreated genomic DNA was also included as a negative control.

**5-Aza-2′-deoxycytidine Assay.** ANXCA cells (10⁶ cells/plate) were treated with culture medium containing the demethylating agent 5-aza-2′-deoxycytidine (2 μM/ml; Sigma-Aldrich, Oakville, Ontario, Canada) for 0, 2, and 4 days with media changed every 2 days. Cells were harvested for DNA using trypsinization and for RNA using the GenElute Mammalian Total RNA Kit (Sigma-Aldrich).

**RT-PCR.** After extraction, RNA was treated with DNase I (1 unit/μg RNA; Sigma-Aldrich) to eliminate any contaminating DNA. First-strand cDNA synthesis (Amersham Pharmacia Biotech, Piscataway, NJ). RT-PCR of APC gene transcripts spanning exons 1A, 1B, 1, 3, and 9 was performed on 5-aza-2′-deoxycytidine-treated and -untreated ANXCA cDNA. Forward primers were designed as follows: 1A-RT-F, 5′-CAGCAGAACAGT-GAGGTTGCTGC-3′ (11); and 1B-RT-F, 5′-GAGGACGAGGAGTGCTG-3′ (14). Reverse primers for exons 1, 3, 5, and 9 were designed as follows: 1B-RT-R, 5′-GTCGAGGGTATATTCTGGAG-3′; 1A-RT-R, 5′-GACCCATAGGAAAGGACTG-3′; 5′-GTCGAGGGTATATTCTGGAGGAGGCTG-3′; and 9-RT-R, 5′-AGCCTTTCTTCTTGCGG-3′. All reactions were carried out at an annealing temperature of 59°C. As a control, RT-PCR of the housekeeping gene β-actin was performed as described previously (15).

**Statistical Analysis.** Statistical analysis was performed using McNemar’s test for paired normal and tumor samples. A matched analysis was used as each tumor sample was compared with normal tissue from the same patient. The association between methylation and MSI + status in tumor samples was analyzed using Fisher’s exact test. Ps <0.05 were considered statistically significant.

**Results and Discussion.** We examined the methylation status of APC promoter 1A in a panel of endometrial cell lines, primary endometrial adenocarcinomas, corresponding hyperplasia, and normal endometrial tissue. Twenty-five percent (29 of 114) of all endometrial adenocarcinomas were methylated with corresponding unmethylated normal tissue (i.e., hypermethylation; Table 1). Using McNemar’s test, this degree of tumor hypermethylation was found to be extremely significant (P < 0.0001). The role of **APC** in endometrial tumorigenesis is poorly understood. Previous studies have described a low frequency of loss of heterozygosity at the **APC** locus (16, 17) while somatic **APC** mutations have never been reported in EC (4). Our results, taken together with these observations, suggest the epigenetic mechanism of promoter methylation rather than somatic mutation is responsible for **APC** inactivation in EC.

In our study, 43% (17 of 40) of MSI + tumors with corresponding unmethylated normal tissue showed hypermethylation compared with 16% (12 of 74) of MSS tumors. Using Fisher’s exact test, **APC** methylation was found to be more significantly associated with MSI + status compared with MSS (P = 0.01). In both groups, the tumor tissue was more likely to show **APC** methylation than the corresponding normal tissue (P = 0.0007 in MSI + cases and P = 0.04 in MSS cases). These results suggest the existence of distinct subtypes of ECs that is recognized by a combination of mismatch repair deficiency and **APC** methylation status. Among the panel of endometrial cell lines examined, ANXCA, HEC-1B, and RL95-2 displayed exclusive methylation of promoter 1A (Fig. 1), whereas the endometrial cell line KLE and colon cell line HT-29 were exclusively unmethylated for both **APC** promoters. Interestingly, all endometrial cell lines with **APC** methylation also display the **MSI** + phenotype (18), whereas the KLE cell line with the **MSI** phenotype (19) was not methylated. Therefore, the endometrial cell line data also reflects our findings of increased **APC** methylation in **MSI** tumors compared with those that are **MSI**. Previous studies have shown **MLH1** hypermethylation to be the principal mechanism underlying development of **MSI** in EC (20). In **MSI** colorectal cancers, a “hypermethylator” phenotype has been proposed, characterized by an increased frequency of global methylation of several candidate genes (21). Our observation of an increased frequency of **APC** methylation in **MSI** + EC is consistent with these findings. Alternatively, increased hypermethylation of **APC** in **MSI** + EC may constitute a molecular mechanism parallel to

**Table 1 APC methylation frequency in MSI + versus MSS tumors**

<table>
<thead>
<tr>
<th>Tumor methylation status</th>
<th>Microsatellite status</th>
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<tbody>
<tr>
<td>Tumor MSS n = 74 (%)</td>
<td>Tumor MSI + n = 40 (%)</td>
</tr>
<tr>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>M</td>
<td>M</td>
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*U, unmethylated; M, methylated.*

**Fig. 1.** A, representative cases normal, hyperplasia, and tumor are shown for MSP of **APC** promoter 1A. Methylation was apparent as early as simple hyperplasia. In B, MSP results for **APC** promoter 1A and 1B are shown in cell lines and control NL DNA. NL DNA treated with SssI methyltransferase was used as a methylated positive control, whereas untreated NL DNA was an unmethylated control. U, unmethylated; M, methylated; NL, normal lymphocyte DNA; N, normal; SH, simple hyperplasia; T, tumor.
β-catenin mutations seen in MSS tumors, both genetic alterations resulting in aberrant activation of the Wnt pathway in a subset of EC. Interestingly, of 114 cases, 5 showed methylation of normal tissue with corresponding unmethylated tumors and 4 exhibited identical methylation profiles of both the tumor and the corresponding normal tissue (Table 1). The significance of APC methylation in normal endometrium, as well as sustained methylation status (i.e., hypomethylation or hypermethylation) among matched normal and tumor pairs, and its implication in EC is not yet known.

Endometrial hyperplasia, specifically atypical hyperplasia, is a premalignant lesion, which may progress to EC in some cases. This progression requires certain genetic changes that occur early and confer a growth advantage during hyperplasia to neoplasia transition. For example, mutations of PTEN, a “gatekeeper” of the endometrium, have been reported in endometrial adenocarcinomas and associated simple, atypical, and complex hyperplastic lesions (22). Of the 29 cases with tumor methylation of APC in our study, endometrial hyperplasia adjacent to tumors was available for 8 MSI+ cases. In 7 of 8 cases, we observed sustained APC promoter 1A methylation in the hyperplasia-carcinoma sequence (Fig. 1). Two cases manifested simple hyperplasia alone, 4 cases showed atypical hyperplasia alone, and there was both simple and atypical hyperplasia in 2 cases. Both simple and atypical endometrial hyperplastic lesions displayed methylation, except in 1 case with atypical hyperplasia alone and in 1 case of both simple and atypical hyperplasia with only the atypical hyperplasia displaying methylation. Our observations of APC methylation in the early precursor lesions suggest that APC methylation may contribute to the development of endometrial tumorigenesis.

APC promoter methylation has been associated with a lack of APC expression in colorectal and pancreatic cancers (11). We treated the endometrial cell line AN3CA, which is exclusively methylated at APC promoter 1A, with the demethylating agent 5-aza-2′-deoxycytidine to determine whether expression of a functional APC protein could be restored. As early as 2 days after treatment with the demethylating agent, MSP analysis indicated an unmethylated APC product (Fig. 2), whereas RT-PCR analysis demonstrated APC transcripts initiated from promoter 1A and spanning exons 1, 3, 5, and 9. As expected, MSP analysis of APC promoter 1B in endometrial cell lines and in a subset of endometrial tumors revealed an absence of methylation (data not shown). These results are consistent with published reports of a lack of promoter 1B methylation in colon, breast, and lung tumors (11).

In EC, despite relative paucity of β-catenin and APC mutations, aberrant Wnt signaling is observed as increased nuclear accumulation of β-catenin (23). Regulation of β-catenin is mediated either by members of the Wnt pathway or via functional interactions with key candidates implicated in other cell signaling pathways. In this context, a recently described role of PTEN in the regulation of β-catenin is particularly intriguing (24). PTEN is shown to induce glycosyn-thase kinase-3β activity, leading to increased phosphorylation of β-catenin and its subsequent degradation. It has also been suggested that PTEN may regulate the translocation of β-catenin from the nucleus but additional studies are necessary to verify this. Furthermore, the frequency and mutational spectra of PTEN alterations are similar in sporadic MSI+ versus MSS ECs (25). Taken together with our observations of APC methylation in both MSI+ and MSS ECs, as well as its occurrence in premalignant lesions, suggest that aberrant Wnt signaling may occur in the early stages of EC.

In summary, we have shown APC methylation in a significant proportion of endometrial tumors and cell lines. Hypermethylation of APC in endometrial tumors is frequently associated with the MSI+ phenotype. Methylation of APC is also observed at the earliest stages of endometrial tumorigenesis, including simple, complex, and atypical hyperplasias. We believe that these findings are significant and suggest that methylation of APC contributes to the development of EC.

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References

11. Esteller, M., Sparks, A., Toyoda, M., Sanchez-Cespedes, M., Capella, G., Peinado, M. A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S. J., Baylin, S. B., and...


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