[CANCER RESEARCH 63, 3101–3106, June 15, 2003]

Multiple Promoters of *Catechol-O-methyltransferase* Gene Are Selectively Inactivated by CpG Hypermethylation in Endometrial Cancer

Masahiro Sasaki, Masanori Kaneuchi, Noriaki Sakuragi, and Rajvir Dahiya[1]

Department of Urology, University of California, San Francisco and Veterans Affairs Medical Center, San Francisco, California 94121 [M. S., M. K., R. D.], and Department of Obstetrics and Gynecology, School of Medicine, Hokkaido University, Kitaku, Sapporo, Japan 060 [N. S.]

ABSTRACT

***Catechol-O-methyltransferase (COMT)* plays an important role in estrogen-induced cancers because *COMT* inactivates catechol estrogens that have cancer-promoting activities. Two promoters control the expression of human *COMT* isoforms: membrane-bound *COMT (MB-COMT)* and soluble *COMT (S-COMT)*. We hypothesize that inactivation of *MB-COMT* and *S-COMT* is important in understanding the pathogenesis of endometrial cancer. To test this hypothesis, we investigated the methylation status and expression of two *COMT* isoforms in 4 endometrial cancer cell lines, 60 endometrial cancer tissues, 10 normal endometrium tissues from normal healthy controls, and 32 pairs of cancerous and normal endometrial samples from the same patients using methylation-specific PCR, methylation-specific sequencing, reverse transcription-PCR, and 5′-rapid amplification of cDNA ends. The results of this study clearly demonstrate that *MB-COMT* was inactivated and methylated, although *S-COMT* was activated and unmethylated in all endometrial cancer cell lines. The 5-aza-2′-deoxycytidine treatment restored *MB-COMT* expression in all cell lines. The promoter for *MB-COMT* was methylated in 47 of 60 cancer tissues but was unmethylated in endometrial tissues from cases without cancer. The promoter for *S-COMT* was unmethylated in all endometrial cancerous and normal tissues. The CpG methylation density at the *MB-COMT* promoter was significantly higher in cancer tissues (a mean of 79.1% of the 19 CpG sites; range, 69–94%) than in adjacent normal tissues (a mean of 8.7% of the 19 CpG sites; range, 3–14%). In summary, these findings demonstrate that methylation of multiple promoters of the *COMT* gene can selectively inactivate *MB-COMT* and may contribute to endometrial carcinogenesis.**

INTRODUCTION

Human endometrial cancer is under the control of estrogens. *COMT*[2] plays an important role in the pathophysiology of many human disorders, including estrogen-related cancers, because the substrates of *COMT* are catechol estrogens (1–3). Catechol estrogens have been reported to represent initiating activity for the carcinogenic process through their direct and indirect ability to cause DNA damage (4–6). Catechol estrogens induce DNA single-strand breaks (7–9). In addition, catechol estrogens have been shown to induce endometrial adenocarcinoma in mice (10). Although estrogens are extensively hydroxylated, catechol estrogen concentrations are low because they are conjugated and metabolized rapidly by *COMT* (5, 11). *COMT* converts catechol estrogens to methoxyestradiols, which are noncarcinogenic metabolites. In fact, methoxyestradiols seem to have potent tumor-suppressing properties *in vitro* (12, 13). These reports strongly suggest that catechol estrogens cause endometrial carcinogenesis and implicate the *COMT* gene as a key player in the process (5, 11).

COMT is expressed in various mammalian tissues, with significant amounts in normal endometrium, ovary, and breast cells (1–4). The *COMT* polypeptides are coded by a single gene assigned to chromosome 22q11.2 (1, 3). Two distinct *COMT*-specific transcripts of 1.3 and 1.5 kb were detected in various human tissues and cell lines. Mapping of the 5′-ends of the *COMT* mRNAs showed two separate sequences (1, 3). MB-ATG and S-ATG codons are responsible for the initiation of translation of the *MB-COMT* and *S-COMT* forms of the enzyme. The proximal promoter (S), located between the two translation initiation codons and extending approximately 200 bp upstream of the MB-ATG initiation codon, gives rise to the 1.3-kb *S-COMT* mRNA. The distal promoter (MB) is located in front of and partly overlapping the transcription start region of the 1.5-kb transcript, controlling the expression of this *MB-COMT* mRNA. *MB-COMT* differs from *S-COMT* polypeptide by extension of an NH_2-terminal 50 amino acids (1, 3, 12). The functional significance of *MB-COMT* and *S-COMT* in different tissues is not known because the lack of *MB-COMT*- and *S-COMT*-specific antibodies has not allowed analysis of the tissue distribution of the two enzyme forms separately (14, 15).

O-Methylation by *COMT* is a major inactivation pathway for catechol estrogens (3, 5, 7). The inhibition of *COMT* in a hamster model enhanced estrogen-induced carcinogenesis and was associated with high catechol estrogen levels (16–18). Various reports observed that the low activity variant of *COMT* was associated with an increased risk for estrogen-related cancers (19–21), although several studies failed to show such a correlation (22, 23). These results suggest that low *COMT* expression and activity contribute to higher concentrations of carcinogenic catechol estrogens that initiate the carcinogenic process. Hypermethylation in CpG islands has been associated with the transcriptional inactivation of the gene and seems to be functionally equivalent to an inactivating mutation for the silencing of these genes (24–26). The *COMT* gene also contains CpG islands in the 5′ upstream region (1, 3, 15). It is not known whether the effects of methylation on multiple promoters in the *COMT* gene lead to equivalent silencing in the levels of *MB-COMT* and *S-COMT* expression in endometrium.

We hypothesize that expression/inactivation of *MB-COMT* and *S-COMT* is important in understanding the pathogenesis of endometrial cancer. To test this hypothesis, we investigated the expression and methylation status of both *MB-COMT* and *S-COMT* in 4 endometrial cancer cell lines and 60 endometrial cancers. MSP and methylation-specific sequencing were used as sensitive methods to detect methylation status for *MB-COMT* and *S-COMT* (27–29). The effect of methylation on the *MB-COMT* and *S-COMT* genes was also studied using the demethylating reagent, 5-azaC, by RT-PCR and 5′-RACE (25). The percentage of methylation for CpG sites was also investigated by methylation-specific sequencing using 32 pairs of cancerous and normal endometrial tissues from the same patients to determine whether *de novo* methylation has occurred in *COMT* isoforms during endometrial carcinogenesis.

Received 8/2/02; accepted 4/11/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Urology Research Center (112F), University of California, San Francisco and Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121. Phone: (415) 750-6964; Fax: (415) 750-6639; E-mail: Urologylab@aol.com.

[2] The abbreviations used are: COMT, catechol-*O*-methyltransferase; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; RACE, rapid amplification of cDNA ends; 5-azaC, 5-aza-2′-deoxycytidine; *MB-COMT*, membrane-bound COMT; *S-COMT*, soluble COMT; FIGO, International Federation of Gynecologists and Obstetricians; ER, estrogen receptor.

MATERIALS AND METHODS

Endometrial Cancer Cell Lines and Primary Cancerous and Normal Endometrial Tissues. Human endometrial cancer cell lines were obtained from American Type Culture Collection. Ishikawa, HHUA, HEC-IB, and MFE-296 were used for these experiments and cultured as described previously (24, 25). The cells were treated with a freshly prepared solution of 5-azaC (Sigma, Santa Cruz, CA). On day 1, a final concentration of 2 μg/ml 5-azaC in PBS was added to the flask. The next day, the medium was changed. On days 3 and 5, the cells were treated with 5-azaC two more times. On day 6, the cells were harvested. Sixty primary cancerous tissues and 10 normal endometrial tissues were obtained from the Department of Gynecology at the Hospital of Hokkaido University (Hokkaido, Japan). The histopathological types of these cancers were as follows: 48 samples of endometrioid cancer; 2 samples of adenosquamous cancer; 3 samples of adenoacantoma; 2 samples of clear cell cancer; and 5 samples of unknown type. In addition, we also used 32 pairs of cancerous and normal endometrial samples from the same patients. Immunohistochemical analysis and Western blotting were not carried out because there are no antibodies specific for *MB-COMT* and *S-COMT*.

DNA Extraction and Sodium Bisulfite Treatment. DNA was isolated from the samples scraped from paraffin-embedded sections. Microdissections were done from these samples as described previously (24, 25). DNA (about 100 ng) was denatured using NaOH and treated with sodium bisulfite for 16 h (Intergen, Purchase, NY) as described previously (24, 25). Modified DNA was resuspended in 50 μl of TE (0.1 M Tris-HCl and 1 mM EDTA) and immediately stored at –20°C.

MSP. Fig. 1 shows the schematic diagram of the COMT gene. The primers and PCR conditions are summarized in Table 1 and Fig. 1. These primer sets are located on each promoter for distinguishing *MB-COMT* and *S-COMT*, separately. The primer sequences were chosen for regions containing frequent cytosines (to distinguish unmodified from modified DNA) and CpG pairs near the 3′-end of primers (24, 25). The fragment of DNA to be amplified was intentionally small because DNA fragments isolated from paraffin sections are generally less than 300-bp long (24, 25). The second-step PCR was performed with two primer sets (Table 1). The first-step PCR was performed with about 10 ng of DNA solution containing 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphate, and 0.5 unit of Taq polymerase (Applied Biosystems Inc., Foster City, CA) in a total volume of 20 μl. The first PCR products (1 μl) were subjected to a second PCR with a second MSP primer set. For MSP, we used one primer set (U) that anneals to unmethylated DNA and another primer set (M) that anneals to methylated DNA.

Quantitation of Methylation Levels by Methylation-specific Sequencing. For confirmation of MSP, the second PCR products by a primer set, MB-SEQ-F and MB-SEQ-R, were purified by QIAquick PCR Purification Kit (Qiagen, Valencia, CA; Table 1). Thirty ng of PCR products were used as a template for sequencing (24, 25). Double-strand sequence analysis was performed using each primer set, an ABI 377 Sequencer, and a Dye Terminator Cycle sequencing kit (Applied Biosystems Inc.). The amount of methylcytosine of each CpG dinucleotide was quantitated by comparing the peak height of the cytosine signal with the peak height of the cytosine plus thymine signal (28). A single cytosine signal at the corresponding CpG site was considered 100% methylation, a single thymine signal was considered no methylation, and overlapping cytosine and thymine signal was considered partial methylation. In the latter instance, the percentage of methylation was expressed as the ratio of the peak value of the cytosine signal to the peak value of the cytosine plus thymine signal.

RNA Isolation, RT-PCR, and 5′-RACE. Four endometrial cell lines and 20 of 60 endometrial cancer tissues were washed and lysed using a guanidine isothiocyanate solution. Total RNA was isolated by phenol-chloroform extraction according to our previous method (25, 27). RT-PCR kits (Perkin-Elmer Corp., Branchburg, NJ) were used to synthesize cDNA from 1.5 μg of total RNA using random hexamer primers. cDNA synthesis was carried out as suggested by the kit protocol, using murine virus reverse transcriptase. Reverse transcription was carried out for 60 min at 42°C. After incubation at 99°C for 5 min to inactivate the reverse transcriptase, the entire 20 μl of cDNA was used to amplify *COMT* isoforms. β-Actin cDNA fragments were also amplified as a positive control (Table 1). Primers for β-actin were chosen specifically to cross two exons in the β-actin gene (24, 25). In the presence of contaminating genomic DNA, additional larger bands would be amplified. The lack of amplification of any larger bands would indicate that there was no contamination with any genomic DNA. Negative controls without RNA and without reverse transcriptase were also performed. *S-COMT* mRNA cannot be distinguished from *MB-COMT* by conventional RT-PCR because *S-COMT* has no specific sequence to distinguish it from *MB-COMT* mRNA. A modified 5′-RACE method was used for accurate evaluation of each mRNA expression (25). RNA (5 μg) was reverse transcribed using a custom-designed 25-mer oligonucleotide, RACE-1ST (5′-TTCTGCTCGCAGTAGGTGTCAATGG-3′). First- and second-strand cDNA synthesis were carried out using RT-PCR kits (Perkin-Elmer Corp.). The cDNA was circularized at 16°C overnight using T4 DNA ligase (Life Technologies, Inc., Grand Island, NY). The circularized cDNA was subjected to PCR using primer

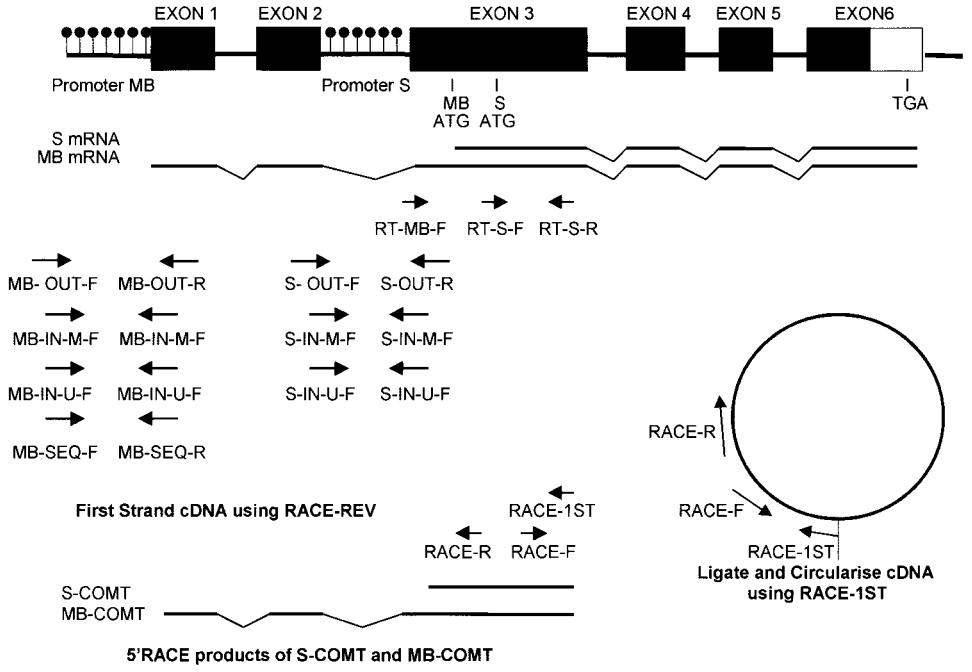

Fig. 1. *A*, structure of the *COMT* gene. The *black lines* represent introns, and *boxes* represent exons. The locations of the MB and S promoters, translation start codons, MB-ATG and S-ATG, and translation stop codon TGA are also noted. *Filled* and *open boxes* correspond to translated and untranslated regions, respectively. CpG sites are shown by *lollipop signs*. Positions and orientation of MSP primers and RT-PCR primers are indicated by *black arrows*. *B*, a schema of modified 5′-RACE method for distinguishing each mRNA expression. mRNA was reverse transcribed using the oligonucleotide RACE-1ST. First- and second-strand cDNA synthesis was carried out. The two different first-strand cDNAs from *MB-COMT* and *S-COMT* are produced by reverse transcription using 5′-RACE-1ST. cDNA was circularized using T4 DNA ligase. The circularized cDNA was subjected to PCR using primer sets RACE-F and RACE-R. By this method, the product from *MB-COMT* had longer bands than that from *S-COMT*.

Table 1 *Summary of the primer sets and PCR conditions for* MB-COMT *and* S-COMT

Primer[a]	Sequence	Length	Denature	Annealing	Extention	Cycle	Final incubation
MB-OUT-F	5′-TATTTGTGGTTAGAAGTAGTT-3′	315 bp	94°C, 30 s	50°C, 60 s	72°C, 60 s	35	72°C, 8 min
MB-OUT-R	5′-AACAACCCTAACTACCCCAA-3′						
MB-IN-M-F	5′-TATTTTGGTTATCGTCGCGC-3′	142 bp	94°C, 30 s	55°C, 30 s	72°C, 60 s	25	72°C, 8 min
MB-IN-M-R	5′-AACGAACGCAAACCGTAACG-3′						
MB-IN-U-F	5′-TATTTTGGTTATTGTTGTGT-3′	142 bp	94°C, 30 s	49°C, 30 s	72°C, 60 s	25	72°C, 8 min
MB-IN-U-R	5′-AACAAACACAAACCATAACA-3′						
MB-SEQ-F	5′-GTAAGATTAGATTAAGAGGT-3′	292 bp	94°C, 30 s	55°C, 45 s	72°C, 60 s	25	72°C, 8 min
MB-SEQ-R	5′-ACAACCCTAACTACCCCAAA-3′						
S-OUT-F	5′-GTTAGGTAATTGAGGTATAA-3′	183 bp	94°C, 30 s	50°C, 60 s	72°C, 60 s	35	72°C, 8 min
S-OUT-R	5′-CCCAACCCCAATACCTCAA-3′						
S-IN-M-F	5′-TTTGCGAATATAAGGGGGC-3′	113 bp	94°C, 30 s	54°C, 45 s	72°C, 60 s	25	72°C, 8 min
S-IN-M-R	5′-CCTCCGACATCTACAAAACG-3′						
S-IN-U-F	5′-TTTGTGAATATAAGGGGGT-3′	113 bp	94°C, 30 s	51°C, 30 s	72°C, 60 s	25	72°C, 8 min
S-IN-U-R	5′-CCTCCAACATCTACAAAACA-3′						
RT-MB-F	5′-ATGCCGGAGGCCCCGCCT-3′	335 bp	94°C, 30 s	59°C, 45 s	72°C, 60 s	27	72°C, 8 min
RT-S-F	5′-GCATCCTGAACCATGTGCTG-3′	162 bp					
RT-R	5′-CATGAACGTGGGCGACAAGAA-3′						
RACE-F	5′-GAGTTCATCCTGCAGCCCAT-3′	430 bp	94°C, 30 s	61°C, 45 s	72°C, 60 s	30	72°C, 8 min
RACE-R	5′-CCAGTGCCTCAGAAGCAGCA-3′	208 bp					
RT-β actin-f	5′-AAGGCCAACCGCGAGAAGAT-3′	147 bp	94°C, 30 s	52°C, 30 s	72°C, 60 s	27	72°C, 8 min
RT-β actin-r	5′-TCGGTGAGGATCTTCATGAG-3′						

[a] MB, *MB-COMT*; S, *S-COMT*; OUT, first PCR; IN, second PCR; F, forward; R, reverse; M, methylated DNA; U, unmethylated DNA; SEQ, sequencing; RT, RT-PCR; RACE, 5′-RACE.

sets RACE-F and RACE-R. By this method, the product from *MB-COMT* had longer band than *S-COMT*.

Statistical Analyses. χ^2 analysis with the Yate's correction was used to determine differences in methylation status of these *COMT* isoforms when compared between endometrial cancerous and normal tissues (24, 25, 27). All statistical tests were two-sided.

RESULTS

Fig. 1 shows the schematic presentation of gene structure of the *COMT* gene (Fig. 1). Positions and orientation of MSP primers and RT-PCR primers are indicated by *black arrows*. Fig. 1 also shows a modified 5′-RACE method. We used a modified 5′-RACE method for distinguishing each mRNA expression because *S-COMT* has no unique sequences compared with *MB-COMT* mRNA. By this method, the product from *MB-COMT* had a longer band than that from *S-COMT*.

Endometrial Cancer Cell Lines. We first determined the expression status of COMT isoforms in four endometrial cancer cell lines [Ishikawa, HHUA, HEC-IB, and MFE-296 (Fig. 2, *A* and *B*, *Lanes 1–4*). In all endometrial cancer cell lines, *MB-COMT* was inactivated

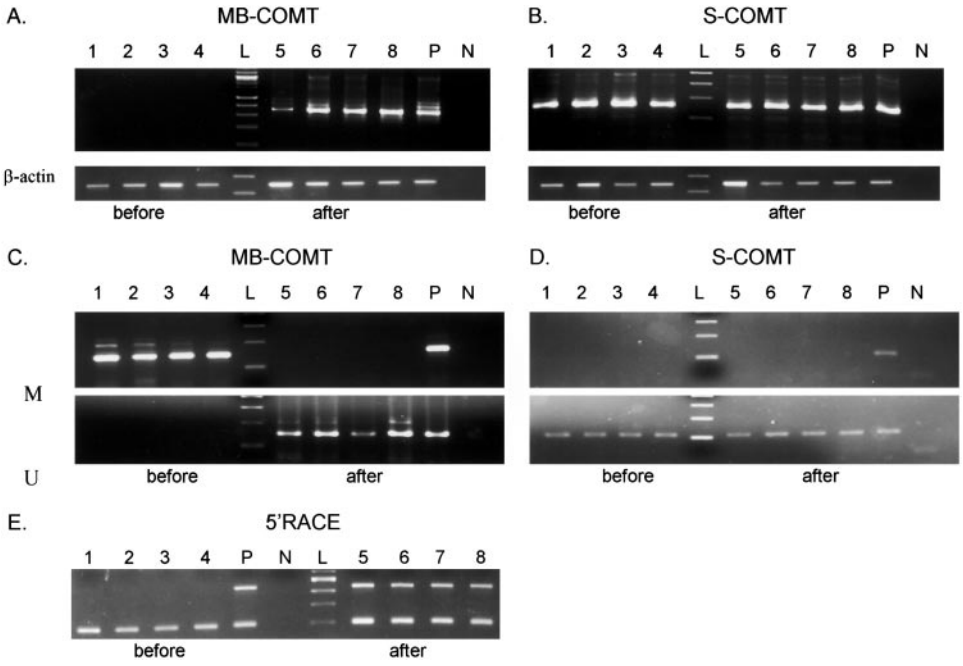

Fig. 2. *A* and *B*, RT-PCR for *MB-COMT* and *S-COMT* in endometrial cancer cell lines Ishikawa, HHUA, HEC-IB, and MFE-296 before and after treatment with demethylating reagent 5-azaC. *Lanes 1–4*, before treatment with 5-azaC; *Lanes 5–8*, after treatment with 5-azaC. *Lanes 1* and *5*, Ishikawa cell line; *Lanes 2* and *6*, HHUA cell line; *Lanes 3* and *7*, HEC-IB cell line; *Lanes 4* and *8*, MFE-296 cell line. *Lane P*, normal human renal cortical epithelial cells (HRCE) for positive control. *Lane N*, negative controls without template RNA. *C* and *D*, methylation status of *MB-COMT* and *S-COMT* in endometrial cancer cell lines Ishikawa, HHUA, HEC-IB, and MFE-296 before and after treatment with 5-azaC. *M*, methylated bands; *U*, unmethylated bands. *Lanes 1–4*, before treatment with 5-azaC; *Lanes 5–8*, after treatment with 5-azaC. *Lanes 1* and *5*, Ishikawa cell line; *Lanes 2* and *6*, HHUA cell line; *Lanes 3* and *7*, HEC-IB cell line; *Lanes 4* and *8*, MFE-296 cell line. *Lane P*, universal methylated DNA (CpGenome Universal Methylated DNA; Intergen, Purchase, NY) for positive control. *Lane N*, negative controls without template DNA. *E*, 5′-RACE for *MB-COMT* and *S-COMT* in endometrial cancer cell lines Ishikawa, HHUA, HEC-IB, and MFE-296 before and after treatment with 5-azaC. *Lanes 1–4*, before treatment with 5-azaC; *Lanes 5–8*, after treatment with 5-azaC. *Lanes 1* and *5*, Ishikawa cell line; *Lanes 2* and *6*, HHUA cell line; *Lanes 3* and *7*, HEC-IB cell line; *Lanes 4* and *8*, MFE-296 cell line. *Lane P*, normal human renal cortical epithelial cells (HRCE) for positive control. *Lane N*, negative controls without template RNA. By 5′-RACE method, longer bands (430 bp) were derived from *MB-COMT* mRNA, and shorter bands (208 bp) were derived from *S-COMT* mRNA.

Table 2 *The methylation status and expressions of* MB-COMT *and* S-COMT *in endometrial cancer tissues*

A. Methylation status

		Cancer tissues		Normal tissues
MB-COMT	Methylated	47/60 (78.3%)	Methylated	0/10 (0%)
	Unmethylated	13/60 (21.7%)	Unmethylated	10/10 (100%)
S-COMT	Methylated	0/60 (0%)	Methylated	0/10 (0%)
	Unmethylated	60/60 (100%)	Unmethylated	10/10 (100%)

B. Expression status

		Expression	
		Positive	Negative
MB-COMT	Methylated	0	15
	Unmethylated	5	0
S-COMT	Methylated	0	0
	Unmethylated	20	0

(Fig. 2*A*, *Lanes 1–4*), but *S-COMT* was activated (Fig. 2*B*, *Lanes 1–4*). We further investigated the methylation status of *COMT* isoforms in these cell lines (Fig. 2, *C* and *D*, *Lanes 1–4*). In all cell lines, the *MB-COMT* promoter was methylated (Fig. 2*C*, *Lanes 1–4*), whereas the *S-COMT* promoter was unmethylated (Fig. 2*D*, *Lanes 1–4*). To investigate the mechanisms of inactivation of *MB-COMT*, we treated these cell lines with the demethylating agent 5-azaC and then analyzed expression by use of MSP and RT-PCR (Fig. 2, *A–D*, *Lanes 5–8*). Demethylation restored expression of *MB-COMT* in all cell lines (Fig. 2*A*, *Lanes 5–8*). Thus, the expression of *COMT* isoforms was related to the methylation status of their corresponding promoters (Fig. 2, *C* and *D*). Fig. 2*E* shows expression of *MB-COMT* and *S-COMT* in these cell lines by 5′-RACE. Only the shorter band derived from *S-COMT* was observed before 5-azaC treatment because of inactivation of *MB-COMT*. However, after 5-azaC treatment, both longer and shorter bands were observed because of reexpression of *MB-COMT*.

Endometrial Cancer Tissues. We analyzed methylation and expression status of two *COMT* isoforms in endometrial cancer tissues and normal tissues (Table 2A). The promoter for *MB-COMT* was methylated in 47 of 60 cancer tissues (78.3%) and unmethylated in all normal tissues ($P < 0.001$). The promoter for *S-COMT* was unmethylated in all endometrial cancer tissues and normal tissues. The correlation between the expression and methylation status of *COMT* isoforms was also investigated using 20 endometrial cancer tissues (Table 2B). No expression of *MB-COMT* was observed in 15 of 20 endometrial cancer tissues in which the promoter for *MB-COMT* was methylated. On the other hand, expression of *MB-COMT* was observed in the other five tissues in which the promoter for *MB-COMT* was unmethylated. Thus, the expression of *MB-COMT* was related to the methylation status of the promoter for *MB-COMT* even in endometrial cancer tissues ($P < 0.001$). *S-COMT* expression was observed, and the promoter for *S-COMT* was unmethylated in all 20 endometrial cancer tissues.

Table 3 shows the association of the methylation status of *MB-COMT* with the classification of FIGO and pathological types (24, 27). *MB-COMT* promoter was methylated in all 23 samples over stage 3 but was unmethylated in 13 of 37 samples under stage 2 ($P < 0.001$).

Methylation-specific Sequencing. We confirmed the results for methylation status of *COMT* isoforms by methylation-specific sequencing in 32 pairs of cancerous and normal endometrial samples from the same cancer patients (Fig. 3). Fig. 3*A* shows the percentage of methylation of *COMT* isoforms on 19 different CpG sites (genomic positions 1362, 1373, 1377, 1379, 1381, 1392, 1394, 1400, 1405, 1425, 1430, 1432, 1435, 1437, 1442, 1449, 1553, 1558, and 1565) of *MB-COMT* in 32 pairs of cancerous and normal tissues from the same cancer patients. The methylation rates of *MB-COMT* at all 19 CpG sites were significantly high in cancer tissues (overall, 79.1%; range, 69–94%), although the rates were generally low in normal tissues (overall, 8.7%; range, 3–14%; Fig. 3*A*). Fig. 3*B* shows a representation of methylation-specific sequencing for a pair of endometrial cancer tissue and normal tissue from the same cancer patient. All cytosines were deaminated and converted to thymines after sodium bisulfite modification for *MB-COMT* in normal tissue (Fig. 3*B*). On the other hand, 5-methylcytosines remained unaltered for *MB-COMT* in the cancer tissue because these 5-methylcytosines were protected by methylation.

DISCUSSION

In the present study, we tested the hypothesis that expression/inactivation of *MB-COMT* and *S-COMT* is important in understanding the pathogenesis of endometrial cancer. The results of these experiments demonstrate that 78.3% of cancer samples had methylated *MB-COMT* alleles, whereas all normal samples had unmethylated *MB-COMT* alleles. *S-COMT* was unmethylated in all cancerous and normal endometrial samples. The promoter for *MB-COMT* was methylated in endometrial cancer tissues in which the expression of *MB-COMT* was inactivated. On the other hand, the promoter for *MB-COMT* was unmethylated in other tissues that showed *MB-COMT* expression. Thus, the expression of *COMT* isoforms was related to the methylation status of the promoters for *COMT* in endometrial cancer tissues.

Table 3 *The methylation status of* MB-COMT *and* S-COMT *in cancer and normal tissues*

FIGO Stage*	Methylated	Unmethylated	Pathology	Methylated	Unmethylated
4b	3/3 (100%)	0/3 (0%)	Endometrioid	37/48 (77.1%)	11/48 (22.9%)
3c	8/8 (100%)	0/8 (0%)	Adenosquamous	2/2 (100%)	0/2 (0%)
3a	12/12 (100%)	0/12 (0%)	Adenoacanthoma	2/3 (66.7%)	1/3 (33.3%)
2b	4/6 (66.7%)	2/6 (33.3%)	Clear cell	2/2 (100%)	0/2 (0%)
1c	8/11 (72.7%)	3/11 (27.3%)	Unknown type	4/5 (80%)	1/5 (20%)
1b	9/14 (64.3%)	5/14 (35.7%)			
1a	3/6 (50%)	3/6 (50%)			

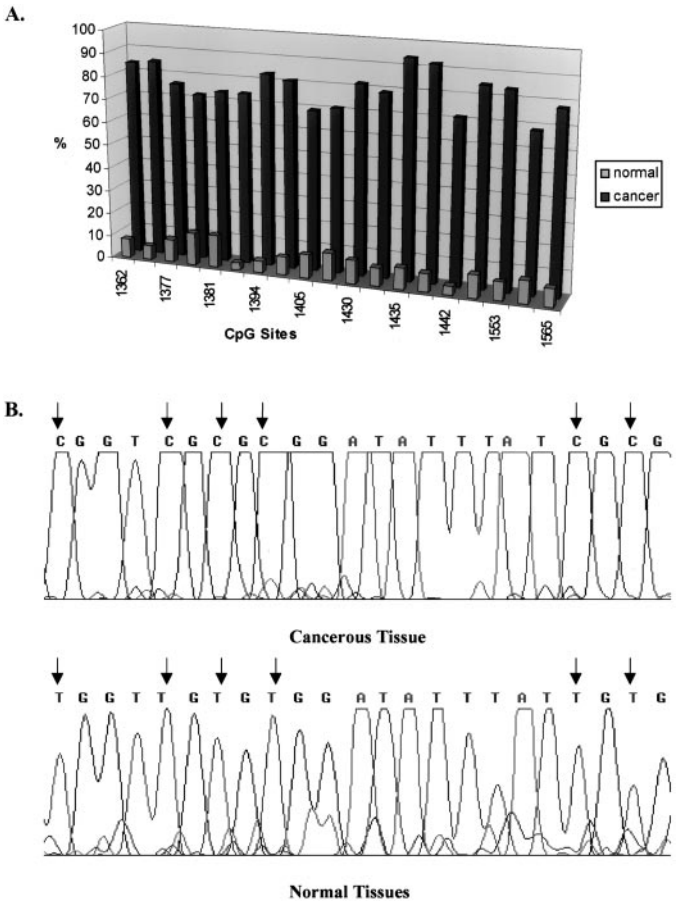

Fig. 3. *A*, the percentage of each CpG island methylation of *MB-COMT* in 32 pairs of cancerous and normal endometrial tissues from the same patients. Nineteen CpG sites (genomic positions 1362, 1373, 1377, 1379, 1381, 1392, 1394, 1400, 1405, 1425, 1430, 1432, 1435, 1437, 1442, 1449, 1453, 1458, and 1465) of *MB-COMT* were analyzed separately in 32 pairs of cancerous and normal tissues. The methylation percentage of each CpG site is presented as the average ratio of methylated cytosine to the total cytosine and thymine of all samples in cancerous and normal endometrial tissues. *Numbers* on the *X* axis are the position of CpG sites. *B*, a representation of MSP sequence for a pair of endometrial cancer and normal tissues from the same patient. All cytosines were completely deaminated and converted to thymines after sodium bisulfite modification in normal tissue. On the other hand, 5-methylcytosines remained unaltered in the cancer tissue because it was protected by methylation. *Arrows* show the methylation status of each CpG site.

The transcription initiation regions of several human genes often contain multiple promoters (24, 25, 29). It is apparent that the multiple promoters to a single gene have a particular role in various cancers (24, 25, 29). For example, the human progesterone receptor contains two promoters (PRA and PRB; Ref. 25). In our recent study, we reported that PRB is strongly inactivated and methylated, whereas PRA is constitutively expressed and unmethylated in endometrial cancer (25). The human ERα also contains three promoters (ERα-A, ERα-B, and ERα-C; Refs. 24 and 29). ERα-A and ERα-B are strongly inactivated and methylated in prostate cancer, whereas only ERα-C is inactivated and methylated in endometrial cancer (24, 29). In the present study, we also found that *COMT* isoforms are selectively inactivated by CpG methylation in endometrial cancer.

The significance of the complicated promoter structure of the *COMT* gene can be interpreted in two ways (24, 25, 29). First, multiple independent promoters should make it possible to control transcription of the gene in a cell type-specific manner, exhibiting multiple functions in various cells (24, 25, 29). Second, the differences in the 5′-region affect the activity of the enzyme (24, 25, 29). *MB-COMT* differs from *S-COMT* polypeptide by having an NH_2-terminal 50-amino acid-long extension (1, 3, 12). It is well documented that *MB-COMT* is more potent than *S-COMT* in detoxification of catechol estrogens (3, 4, 13, 15, 30). When *MB-COMT* is inactivated, it will lead to accumulation of catechol estrogens and promote carcinogenesis. In the present study, *MB-COMT* expression was inactivated through CpG methylation, which may lead to higher levels of catechol estrogens.

Inactivation of catechol estrogens may be especially important for the endometrium because of the potential carcinogenicity and the estrogenic activity of catechol estrogens (31–33). When metabolic clearance of the catechol estrogen is slow or incomplete, reactive oxygen species may accumulate and possibly promote the development of endometrial cancer (5, 6, 10, 33, 34). In this regard, prior studies have shown that inhibition of *COMT* by quercetin in rodents can enhance the appearance of hormone-inducible tumors (13). Abnormal methylation has been observed for several genes in cancer cells. Such methylation has recently been identified as an alternate mechanism of inactivating tumor suppressor genes during the development of cancer (24–29). It has been suggested that *COMT* might protect the endometrial tissue against the carcinogenic effects of estrogen metabolites (12, 13). Thus, it is possible that inactivation of *COMT* reduces the ability to prevent cytotoxic and genotoxic damage caused by products of catechol estrogens (12, 13).

In the present study, we found *MB-COMT* to be selectively inactivated and methylated in endometrial cancer cell lines and tissues. In contrast, *S-COMT* was expressed and unmethylated. This is the first report demonstrating that selective methylation of the *COMT* gene induces inactivation of more active form, *MB-COMT*, and may contribute to endometrial carcinogenesis.

REFERENCES

1. Lundstrom, K., Tenhunen, J., Tilgmann, C., Karhunen, T., Panula, P., and Ulmanen, I. Cloning, expression and structure of catechol-*O*-methyltransferase. Biochim. Biophys. Acta, *1251:* 1–10, 1995.
2. Ulmanen, I., Peranen, J., Tenhunen, J., Tilgmann, C., Karhunen, T., Panula, P., Bernasconi, L., Aubry, J. P., and Lundstrom, K. Expression and intracellular localization of catechol *O*-methyltransferase in transfected mammalian cells. Eur. J. Biochem., *243:* 452–459, 1997.
3. Bertocci, B., Miggiano, V., Da Prada, M., Dembic, Z., Lahm, H. W., and Malherbe, P. Human catechol-*O*-methyltransferase: cloning and expression of the membrane-associated form. Proc. Natl. Acad. Sci. USA, *88:* 1416–1420, 1991.
4. Tenhunen, J., Heikkila, P., Alanko, A., Heinonen, E., Akkila, J., and Ulmanen, I. Soluble and membrane-bound catechol-*O*-methyltransferase in normal and malignant mammary gland. Cancer Lett., *144:* 75–84, 1999.
5. Yager, J. D., and Liehr, J. G. Molecular mechanisms of estrogen carcinogenesis. Annu. Rev. Pharmacol. Toxicol., *266:* 203–232, 1996.
6. Li, J. J., Li, S. A., Klicka, J. K., Parsons, J. A., and Lam, L. K. Relative carcinogenic activity of various synthetic and natural estrogens in the Syrian hamster kidney. Cancer Res., *43:* 5200–5204, 1983.
7. Cavalieri, E. L., Stack, D. E., Devanesan, P. D., Todorovic, R., Dwivedy, I., Higginbotham, S., Johansson, S. L., Patil, K. D., Gross, M. L., Gooden, J. K., Ramanathan, R., Cerny, R. L., and Rogan, E. G. Molecular origin of cancer: catechol estrogen-3, 4-quinones as endogenous tumor initiators. Proc. Natl. Acad. Sci. USA, *94:* 10937–10942, 1997.
8. Cavalieri, E., Frenkel, K., Liehr, J. G., Rogan, E., and Roy, D. Estrogens as endogenous genotoxic agents-DNA adducts and mutations. J. Natl. Cancer Inst. Monogr., *2000:* 75–93, 2000.
9. Hiraku, Y., Yamashita, N., Nishiguchi, M., and Kawanishi, S. Catechol estrogens induce oxidative DNA damage and estradiol enhances cell proliferation. Int. J. Cancer, *92:* 333–337, 2001.
10. Newbold, R. R., and Liehr, J. G. Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. Cancer Res., *60:* 235–237, 2000.
11. Xie, T., Ho, S. L., and Ramsden, D. Characterization and implications of estrogenic down-regulation of human catechol-*O*-methyltransferase gene transcription. Mol. Pharmacol., *56:* 31–38, 1999.
12. Mannisto, P. T., and Kaakkola, S. Catechol-*O*-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacol. Rev., *51:* 593–628, 1999.
13. Zhu, B. T., and Liehr, J. G. Inhibition of catechol *O*-methyltransferase-catalyzed *O*-methylation of 2- and 4-hydroxyestradiol by quercetin. Possible role in estradiol-induced tumorigenesis. J. Biol. Chem., *271:* 1357–1363, 1996.
14. Inoue, K., Tice, L. W., and Creveling, C. R. Immunocytochemical localization of catechol-*O*-methyltransferase in the pregnant rat uterus. Endocrinology, *107:* 1833–1840, 1980.

15. Tenhunen, J., Salminen, M., Lundstrom, K., Kiviluoto, T., Savolainen, R., and Ulmanen, I. Genomic organization of the human catechol *O*-methyltransferase gene and its expression from two distinct promoters. Eur. J. Biochem., *223:* 1049–1059, 1994.
16. Liehr, J. G., and Sirbasku, D. A. Estrogen-dependent kidney tumors. *In:* M. Taub (ed.), Tissue Culture of Epithelial Cells, pp. 205–234. New York: Plenum, 1985.
17. Li, J. J., Hou, X., Bentel, J., Yazlovitskaya, E. M., and Li, S. A. Prevention of estrogen carcinogenesis in the hamster kidney by ethinylestradiol: some unique properties of a synthetic estrogen. Carcinogenesis (Lond.), *19:* 471–477, 1998.
18. Jefcoate, C. R., Liehr, J. G., Santen, R. J., Sutter, T. R., Yager, J. D., Yue, W., Santner, S. J., Tekmal, R., Demers, L., Pauley, R., Naftolin, F., Mor, G., and Berstein, L. Tissue-specific synthesis and oxidative metabolism of estrogens. J. Natl. Cancer Inst. Monogr., *2000:* 95–112, 2000.
19. Thompson, P. A., Shields, P. G., Freudenheim, J. L., Stone, A., Vena, J. E., Marshall, J. R., Graham, S., Laughlin, R., Nemoto, T., Kadlubar, F. F., and Ambrosone, C. B. Genetic polymorphisms in catechol-*O*-methyltransferase, menopausal status, and breast cancer risk. Cancer Res., *58:* 2107–2110, 1998.
20. Yim, D. S., Parkb, S. K., Yoo, K. Y., Yoon, K. S., Chung, H. H., Kang, H. L., Ahn, S. H., Noh, D. Y., Choe, K. J., Jang, I. J., Shin, S. G., Strickland, P. T., Hirvonen, A., and Kang, D. Relationship between the Val158Met polymorphism of catechol *O*-methyl transferase and breast cancer. Pharmacogenetics, *11:* 279–286, 2001.
21. Huang, C. S., Chern, H. D., Chang, K. J., Cheng, C. W., Hsu, S. M., and Shen, C. Y. Breast cancer risk associated with genotype polymorphism of the estrogen-metabolizing genes CYP17, CYP1A1, and COMT: a multigenic study on cancer susceptibility. Cancer Res., *59:* 4870–4875, 1999.
22. Hamajima, N., Matsuo, K., Tajima, K., Mizutani, M., Iwata, H., Iwase, T., Miura, S., Oya, H., and Obata, Y. Limited association between a catechol-*O*-methyltransferase (COMT) polymorphism and breast cancer risk in Japan. Int. J. Clin. Oncol., *6:* 13–18, 2001.
23. Millikan, R. C., Pittman, G. S., Tse, C. K., Duell, E., Newman, B., Savitz, D., Moorman, P. G., Boissy, R. J., and Bell, D. A. Catechol-*O*-methyltransferase and breast cancer risk. Carcinogenesis (Lond.), *19:* 1943–1947, 1998.
24. Sasaki, M., Kotcherguina, I., Dharia, A., Fujimoto, S., and Dahiya, R. Cytosine-phosphoguanine methylation of estrogen receptors in endometrial cancer. Cancer Res., *61:* 3262–3266, 2001.
25. Sasaki, M., Oh, B. R., Dharia, A., Fujimoto, S., and Dahiya, R. Progesterone receptor B gene inactivation and CpG hypermethylation in human uterine endometrial cancer. Cancer Res., *61:* 97–102, 2001.
26. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.
27. Sasaki, M., Oh, B. R., Dharia, A., Fujimoto, S., and Dahiya, R. Inactivation of human androgen receptor gene is associated with CpG hypermethylation in uterine endometrial cancer. Mol. Carcinog., *29:* 59–66, 2000.
28. Li, L. C., Chui, R., Nakajima, K., Oh, B. R., Au, H. C., and Dahiya, R. Frequent methylation of estrogen receptor in prostate cancer: correlation with tumor progression. Cancer Res., *60:* 702–706, 2000.
29. Sasaki, M., Tanaka, Y., Perinchery, G., Dharia, A., Kotcherguina, L., Fujimoto, S., and Dahiya, R. Methylation and inactivation of estrogen, progesterone, and androgen receptors in prostate cancer. J. Natl. Cancer Inst. (Bethesda), *94:* 384–390, 2002.
30. Malherbe, P., Bertocci, B., Caspers, P., Zurcher, G., and Da Prada, M. Expression of functional membrane-bound and soluble catechol-*O*-methyltransferase in *Escherichia coli* and a mammalian cell line. J. Neurochem., *58:* 1782–1789, 1992.
31. Henderson, B. E., and Feigelson, H. S. Hormonal carcinogenesis. Carcinogenesis (Lond.), *21:* 427–433, 2000.
32. Zhu, B. T., Roy, D., and Liehr, J. G. The carcinogenic activity of ethinyl estrogens is determined by both their hormonal characteristics and their conversion to catechol metabolites. Endocrinology, *132:* 577–583, 1993.
33. Liehr, J. G., Ricci, M. J., Jefcoate, C. R., Hannigan, E. V., Hokanson, J. A., and Zhu, B. T. 4-Hydroxylation of estradiol by human uterine myometrium and myoma microsomes: implications for the mechanism of uterine tumorigenesis. Proc. Natl. Acad. Sci. USA, *92:* 9220–9224, 1995.
34. Kennedy, J. A. Effect of catechol oestrogens on extraneuronal metabolism of noradrenaline by rabbit uterine endometrium and myometrium. Naunyn. Schmiedebergs. Arch. Pharmacol., *343:* 266–270, 1991.