Multiple Promoters of \textit{Catechol-O-methyltransferase} Gene Are Selectively Inactivated by CpG Hypermethylation in Endometrial Cancer

Masahiro Sasaki, Masanori Kaneuchi, Noriaki Sakuragi, and Rajvir Dahiya\textsuperscript{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.]

\textbf{ABSTRACT}

\textit{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 \textit{human COMT} (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 \textit{COMT} isoforms in \textit{four} endometrial cancer cell lines, \textit{60} endometrial cancer tissues, \textit{10} normal endometrium tissues from \textit{normal} human controls, and \textit{32} pairs of \textit{cancerous} and \textit{normal} endometrial samples from the same patients using \textit{methylation-specific} PCR, \textit{methylation-specific} sequencing, \textit{reverse transcription-PCR}, and \textit{5-azaC}-rapid amplification of \textit{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 \textit{47} of \textit{60} cancer tissues but was unmethylated in endometrial tissues from \textit{cases} without cancer. The promoter for S-COMT was unmethylated in all endometrial cancerous and normal tissues. The \textit{CpG} methylation density at the MB-COMT promoter was significantly higher in cancer tissues (a mean of 79.1% of the 19 \textit{CpG} sites; range, 69–94%) than in adjacent normal tissues (a mean of 8.7% of the 19 \textit{CpG} sites; range, 3–14%). In summary, these findings demonstrate that methylation of multiple promoters of the \textit{COMT} gene can selectively inactivate MB-COMT and may contribute to endometrial carcinogenesis.

\textbf{INTRODUCTION}

Human endometrial cancer is under the control of estrogens. COMT\textsuperscript{2} plays an important role in the pathophysiology of many human disorders, including estrogen-related cancers, because the sub-strates 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 methoxyxestriadiols, which are noncarcinogenic metabolites. In fact, methoxyxestriadiols seem to have potent tumor-suppressing properties \textit{in vitro} (12, 13). These reports strongly suggest that catechol estrogens cause endometrial carcinogenesis and implicate the \textit{COMT} gene as a key player in the process (5, 11).

\textit{COMT} is expressed in various mammalian tissues, with significant amounts in normal endometrium, ovary, and breast cells (1–4). The \textit{COMT} polypeptides are coded by a single gene assigned to chromosome 22q11.2 (1, 3). Two distinct \textit{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 \textit{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\textsubscript{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).

\textit{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 \textit{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 \textit{COMT} expression and activity contribute to higher concentrations of carcinogenic catechol estrogens that initiate the carcinogenic process. Hypermethylation in \textit{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 \textit{COMT} gene also contains \textit{CpG} islands in the 5′ upstream region (1, 3, 15). It is not known whether the effects of methylation on multiple promoters in the \textit{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 \textit{four} endometrial cancer cell lines and \textit{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 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 \textit{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 \textit{de novo} methylation has occurred in \textit{COMT} isoforms during endometrial carcinogenesis.
MATERIALS AND METHODS

Endometrial Cancer Cell Lines and Primary Cancers and Normal Endometrial Tissues. Human endometrial cancer cell lines were obtained from American Type Culture Collection. Ishikawa, HUCA, HECA, 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 characteristics of these cancers were as follows: 48 samples of endometrioid cancer; 2 samples of adenosquamous cancer; 3 samples of adenocarcinoma; 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 translation stop codon TGA. By this method, the product from 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 characteristics of these cancers were as follows: 48 samples of endometrioid cancer; 2 samples of adenosquamous cancer; 3 samples of adenocarcinoma; 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.

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 filled bars. 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.

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′-TTCTGCTCCGACGATGTTGTCATATG-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 sets RACE-F and RACE-R. By this method, the product from MB-COMT had longer bands than that from S-COMT.
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**Statistical Analyses.** χ² 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 before treatment with 5-azaC; Lanes 2, after 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 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cancer tissues</th>
<th>Normal tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB-COMT</td>
<td>Methylated</td>
<td>47/60 (78.3%)</td>
</tr>
<tr>
<td></td>
<td>Unmethylated</td>
<td>13/60 (21.7%)</td>
</tr>
<tr>
<td>S-COMT</td>
<td>Methylated</td>
<td>0/60 (0%)</td>
</tr>
<tr>
<td></td>
<td>Unmethylated</td>
<td>60/60 (100%)</td>
</tr>
</tbody>
</table>

B. Expression status

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB-COMT</td>
<td>Methylated</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>S-COMT</td>
<td>Unmethylated</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Methylated</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Unmethylated</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

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 isomers was related to the methylation status of the promoters for COMT in endometrial cancer tissues.
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 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, MB-COMT expression 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


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