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Cytosine-Phosphoguanine Methylation of Estrogen Receptors in Endometrial Cancer

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Abstract

We hypothesize that estrogen receptors (ERs) are differentially expressed in endometrial cancer. To test this hypothesis, we investigated the expression profile of ERα (ERα-A, ERα-B, ERα-C) and ERβ genes and CpG methylation status in endometrial cancer cell lines and tissues using reverse transcription-PCR and methylation-specific PCR and direct DNA sequencing. The results demonstrated that ERα-A, ERα-B, and ERβ were normally expressed whereas ERα-C gene was inactivated in all endometrial cancer cell lines. We further investigated the mechanisms of ERα-C gene inactivation through CpG methylation pathways. The treatment with demethylating agent (5′-aza-2′-deoxycytidine) restored ERα-C gene expression in all endometrial cancer cell lines. We further confirmed these findings with methylation-specific PCR and direct DNA sequencing and found that only ERα-C was methylated on all five different CpG sites in all cell lines. We further analyzed 88 cancerous and 46 normal endometrial tissues. The results demonstrated that only ERα-C was inactivated and methylated in 94% of cancer tissues. In 32 pairs of cancerous and normal endometrial tissues from the same patient, ERα-C was methylated in 29 of 32 cancer tissues but unmethylated in all normal endometrial tissues. This is the first report that demonstrates selective ERα-C gene inactivation through CpG methylation pathway in uterine endometrial cancer.

Introduction

Estrogens exert their effects through two ER[2] types (α and β) (1, 2). Three promoters, A, B, and C, have been identified for the human ERα gene (3, 4). These promoters regulate the synthesis of specific transcripts corresponding to ERα-A, ERα-B, and ERα-C isoforms. These ERs differ in various types of cells and tissues (3, 4). The expression levels of these ERs differ with respect to each other in certain target samples. The specific role of these ERs is unclear; however, the existence of elaborate mechanisms regulating their production suggests that each ER has a specific character. The ERs in specific cells may be critical for appropriate cellular response to estrogen. This specific pattern of ERs expression may enable estrogens to direct their effects to target tissues. Until now, there have been very few reports about mutation or other structural alterations of the ER genes in endometrial cancer (5, 6). One possible mechanism for changing the transcriptional status is methylation of CpG-enriched regions in this gene (7, 8). The ER genes contain CpG-enriched regions in the 5′-upstream region (9, 10). ERα gene methylation has been observed in several human cancers such as breast (11), lung (12), colorectal (13), and hematopoietic neoplasm (14) and has been related to inactivation of ER gene expression (15). However, the expression profile of ERα (ERα-A, ERα-B, ERα-C) and ERβ and their CpG methylation status in endometrial cancer is lacking. This question is critical in understanding the complexity of ER gene expression and regulation in endometrial cancer cells. In this report, we analyzed the expression and methylation status of three ERα isoforms (ERα-A, ERα-B, and ERα-C) and ERβ genes in endometrial cancer cell lines and tissues.

Materials and Methods

Tissue Samples and Cell Lines. Ishikawa, HHUA, HEC-IB, and MFE-296 endometrial cell lines were maintained in DMEM containing 10% FCS. The cells were treated with a freshly prepared solution of 5-aza-2′-deoxycytidine (Sigma Chemical Co., St. Louis, MO; Santa Cruz Biotechnology, 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 twice more as on day 1. On day 6, the cells were harvested (16).

Primary endometrial tissues were obtained from the Department of Gynecology at the hospital of Hokkaido University, Japan. DNA was obtained from 88 cancer and 46 normal uterine endometrial samples. The histopathological types of the cancers were: 67 samples, endometrioid cancer; 2 samples, adenosquamous cancer; 5 samples, adenoacanthoma; 2 samples, clear cell cancer; and 12 samples, unknown type. In addition, we used 32 pairs of cancerous and normal endometrial samples from the same endometrial cancer patient.

RNA Isolation and RT-PCR. Cells were washed and lysed with guanidine isothiocyanate solution. Total RNA was isolated by our previous method (16). Three sets of primers, RT-A, RT-B, and RT-C, specifically amplify transcripts originating from promoters A, B, and C, respectively. A set of primers (RT-Common-f and RT-Common-r) amplifies transcripts common to these promoters. A set of primers (ERβ-f and RT-ERβ-r) specifically amplifies transcripts of ERβ. Primers for β-actin were chosen specifically to cross one intron in the β-actin gene. In the presence of contaminating genomic DNA, additional larger bands would be amplified; the lack of amplification of the larger band was used as a control to rule out contamination with any genomic DNA. Negative controls without RNA and without reverse transcriptase were also performed.

DNA Extraction and Sodium Bisulfite Treatment. DNA was isolated from the samples scraped from the paraffin-embedded sections. The microdissections were done from these samples as described previously (16). DNA (~100 ng) was denatured using NaOH and treated with sodium bisulfite for 16 h (Introgen) as described previously (16). Modified DNA was resuspended in 50 μl of Tris-EDTA and immediately stored at −20°C.

MSP and DNA Sequencing. The primers and their PCR conditions are summarized in Table 1 and Fig. 1. The fragment of DNA to be amplified was intentionally small for application of this technique to paraffin blocks, in which amplification of larger fragments is not possible. One primer set (U) will anneal to unmethylated DNA. A second primer set (M) will anneal to methylated DNA. The methods of PCR, electrophoresis, and gel visualization were described previously (16). For confirmation of MSP, the PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and applied to direct DNA sequencing (16).

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[2] The abbreviations used are: ER, estrogen receptor; 5-azaC, 5-aza-2′-deoxycytidine; MSP, methylation-specific PCR.

Table 1 *Summary of the primer sets and PCR conditions for ERα and ERβ*

Primer	Sequence	Denature	Annealing	Extension	Cycle	Final incubation
ERα-A-Wf ERα-A-Wr	5′-GGACACGGTCTGCACCTGCCCGC-3′ 5′-GCGGACGGTTGAGGGGCTCCAGCT-3′	94°C, 30 s	59°C, 30 s	72°C, 60 s	40	72°C, 8 min
ERα-A-Uf ERα-A-Ur	5′-GGATATGGTTTGTATTTTGTTTGT-3′ 5′-ACAAACAATTCAAAAACTCCAACT-3′	94°C, 30 s	46°C, 30 s	72°C, 60 s	40	72°C, 8 min
ERα-A-Mf ERα-A-Mr	5′-GATACGGTTTGTATTTTGTTCGC-3′ 5′-CGAACGATTCAAAAACTCCAACT-3′	94°C, 30 s	49°C, 45 s	72°C, 60 s	40	72°C, 8 min
ERα-B-Wf ERα-B-Wr	5′-CCCACTGCCATTCATCCAGC-3′ 5′-AGGAATGTGCTCGCATGTGCG-3′	94°C, 30 s	57°C, 30 s	72°C, 60 s	40	72°C, 8 min
ERα-B-Uf ERα-B-Ur	5′-TTTATTGTTATTTATTTAGT-3′ 5′-AAAAATATACTCACATATACA-3′	94°C, 30 s	45°C, 30 s	72°C, 60 s	40	72°C, 8 min
ERα-B-Mf ERα-B-Mr	5′-TTTATTGTTATTTATTTAGC-3′ 5′-AAAAATATACTCGCATATACG-3′	94°C, 30 s	51°C, 45 s	72°C, 60 s	40	72°C, 8 min
ERα-C-Wf ERα-C-Wr	5′-CTTCACATTCTCCGGGACTGC-3′ 5′-GAAGGCTCAGAAACCGGCG-3′	94°C, 30 s	59°C, 30 s	72°C, 60 s	40	72°C, 8 min
ERα-C-Uf ERα-C-Ur	5′-TTTTATATTTTTTGGGATTGT-3′ 5′-AAAAACTCAAAAACCAACA-3′	94°C, 30 s	46°C, 30 s	72°C, 60 s	40	72°C, 8 min
ERα-C-Mf ERα-C-Mr	5′-TTTTATATTTTTCGGGATTGC-3′ 5′-AAAAACTCAAAAACCGGCG-3′	94°C, 30 s	49°C, 45 s	72°C, 60 s	40	72°C, 8 min
ERβ-Wf ERβ-Wr	5′-CTTGGAAGGTGGGCCTGGTC-3′ 5′-CGCATACAGATGTGATAACTGGCG-3′	94°C, 30 s	59°C, 30 s	72°C, 60 s	40	72°C, 8 min
ERβ-Uf ERβ-Ur	5′-TTTGGAAGGTGGGTTTGGTT-3′ 5′-CACATACAAATATAATAACTAACA-3′	94°C, 30 s	46°C, 30 s	72°C, 60 s	40	72°C, 8 min
ERβ-Mf ERβ-Mr	5′-TTTGGAAGGTGGGTTTGGTC-3′ 5′-CGCATACAAATATAATAACTAACG-3′	94°C, 30 s	49°C, 45 s	72°C, 60 s	40	72°C, 8 min
RT-C	5′-GCACAGCACTTCTTGAAAAAGG-3′	94°C, 30 s	59°C, 45 s	72°C, 60 s	40	72°C, 8 min
RT-B	5′-CACATGCGAGCACATTCCTTCC-3′	94°C, 30 s	61°C, 45 s	72°C, 60 s	40	72°C, 8 min
RT-A	5′-CCTCGGGCTGTGCTCTTTTTCC-3′	94°C, 30 s	62°C, 45 s	72°C, 60 s	40	72°C, 8 min
RT-r	5′-AGGGTCATGGTCATGGTCCG-3′					
RT-Common-f RT-Common-r	5′-ACGACTATATGTCCAGCC-3′ 5′-AGGTTGGCAGCTCTCATGTCTCC-3′	94°C, 30 s	62°C, 45 s	72°C, 60 s	40	72°C, 8 min
RT-ERβ-f RT-ERβ-r	5′-CTTGGAAGGTGGGCCTGGTC-3′ 5′-CGCATACAGATGTGATAACTGGCG-3′	94°C, 30 s	59°C, 30 s	72°C, 60 s	40	72°C, 8 min
β-Actin-f β-Actin-r	5′-AAGGCCAACCGCGAGAAGAT-3′ 5′-TCGGTGAGGATCTTCATGAG-3′	94°C, 30 s	52°C, 30 s	72°C, 60 s	40	72°C, 8 min

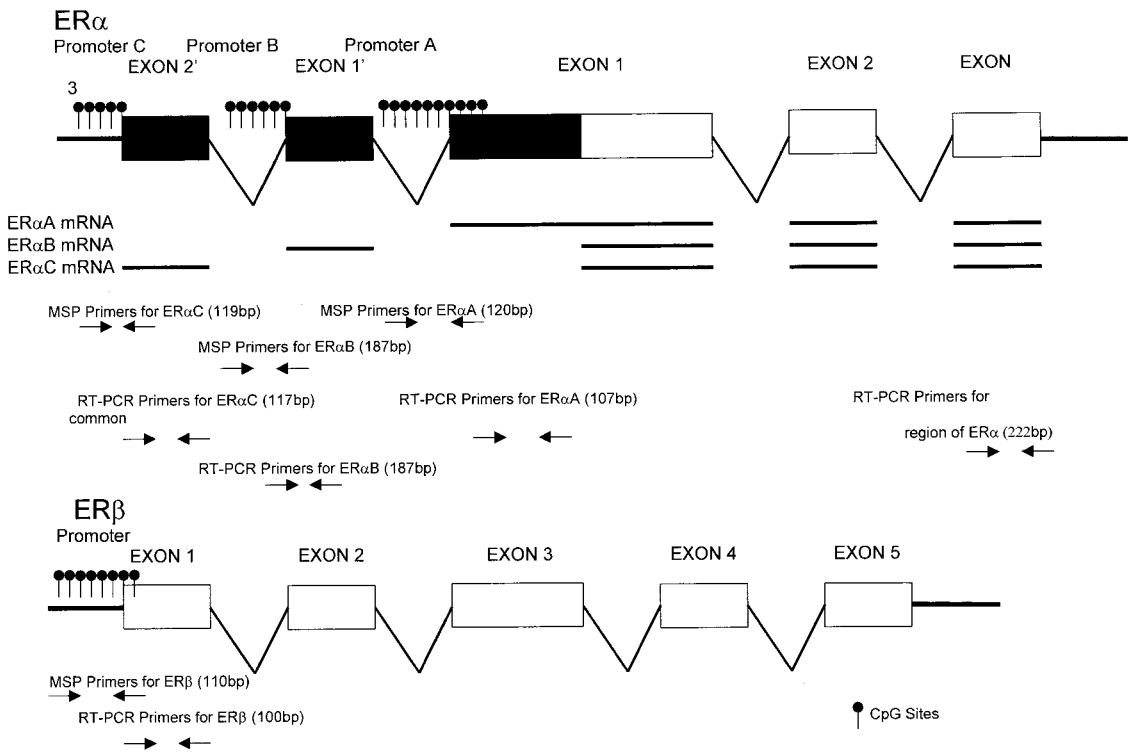

Fig. 1. Structure of the upstream region of ERα and ERβ genes. The genomic DNA ER types are schematically represented and three ERα promoters are shown in Fig. 1. *Bold black lines*, positions and orientations for these MSP products; *thin black lines*, those of RT-PCR primers; ■, specific regions to these isoforms in exon 1; *lollipop signs*, CpG sites.

Table 2 *Expression profiles of ER in cell lines treated or not with 5-azaC*

	ERα-A		ERα-B		ERα-C		ERβ		
	Methylation	Expression	Methylation	Expression	Methylation	Expression	Methylation	Expression	β-Actin
Before treatment									
Ishikawa	−	+	−	+	+	−	−	+	+
HHUA	−	+	−	+	+	−	−	+	+
HEC-1B	−	+	−	+	+	−	−	+	+
MFE-296	−	+	−	+	+	−	−	+	+
After treatment									
Ishikawa	−	+	−	+	−	+	−	+	+
HHUA	−	+	−	+	−	+	−	+	+
HEC-1B	−	+	−	+	−	+	−	+	+
MFE-296	−	+	−	+	−	+	−	+	+

Results

Cell Lines. MSP and RT-PCR were performed using these cell lines treated with and without demethylating reagent, 5-azaC (Table 2, Fig. 2). By RT-PCR without 5-azaC treatment, no ERα-C expression were found, although ERα-A, ERα-B, and ERβ expression was found in all of endometrial cancer cell lines examined (Fig. 2). By MSP, only ERα-C was methylated in all of the cell lines, whereas others were unmethylated. Treatment of cell lines with 5-azaC restored ERα-C expression in all of cancer cell lines (Table 2, Fig. 2).

Cancerous and Normal Endometrial Tissues. All of the ERs were unmethylated in all 46 normal endometrial tissues (Table 3A). ERα-C was methylated in 83 of 88 cancer tissues (94%), whereas ERα-A, ERα-B, and ERβ were unmethylated in all 88 cancerous tissues. A significant difference in the methylation status in ERα-C was found between cancerous and normal endometrial tissues ($P < 0.001$). Table 3, B and C, shows the correlation of the methylation status of ERα-C, clinical stage, and pathological types of cancer tissues. All cancer tissues over stage 3 were methylated, whereas 5 of 58 cancer samples under stage 2 were unmethylated ($P < 0.01$).

Pairs of Cancerous and Normal Samples. ERα-A, ERα-B, and ERβ were unmethylated in all 32 pairs of cancerous and normal tissues. ERα-C was methylated in 29 of 32 cancer tissues (91%) and unmethylated in all 32 normal tissues ($P < 0.001$; Table 4). Fig. 3*A* shows typical results of the MSP assay of these ERα isoforms and ERβ in a pair of cancer and normal tissues from the same patient. Only the unmethylated bands of ERα-A, ERα-B, and ERβ were observed in both cancerous and normal tissue (*Lanes 1–8* and *13–16*). There were only methylated ERα-C bands in cancerous tissues (*Lanes 11* and *12*), although only unmethylated ERα-C bands was observed in normal tissues (*Lanes 9* and *10*). Fig. 3*B* shows the sequence data of ERα-C from these alleles. All CpG sites of ERα-C were methylated in endometrial cancer cell lines and cancerous tissues. All C are deaminated and converted to T of ERα-C in the normal tissue, whereas 5-methylcytosines remain unaltered in the cancer tissue, because it is methylated (Fig. 3*B*).

Discussion

ER are implicated in the control of proliferation, differentiation, and development of uterine endometrial cells. The ERs are members of a closely related subgroup of nuclear receptors that includes the androgen, mineral corticoid, and glucocorticoid receptors (1, 17). Although ERs have three ERα isoforms and ERβ genes, the biological significance of these isoforms and subtypes is unclear (2–4). The discovery of three promoters in the ERα gene has previously led us to suggest that differential promoter usage could be one mechanism regulating expression of this steroid hormone receptor (4, 17).

In this study, we investigated the expression of three isoforms of ERα (ERα-A, ERα-B, and ERα-C) and ERβ in endometrial cancer cell lines. We found that only the transcript from the distal promoter, ERα-C, is inactivated in human endometrial cancer cells, whereas both transcripts, ERα-A, and ERα-B, are present in endometrial cancer. We also investigated the mechanisms of inactivation of ERα-C gene through the analysis of CpG methylation using MSP and direct DNA sequencing. Only ERα-C is methylated in all human endometrial cancer cells, whereas other cells are unmethylated. The treatment of endometrial cancer cells with 5-azaC restored ERα-C

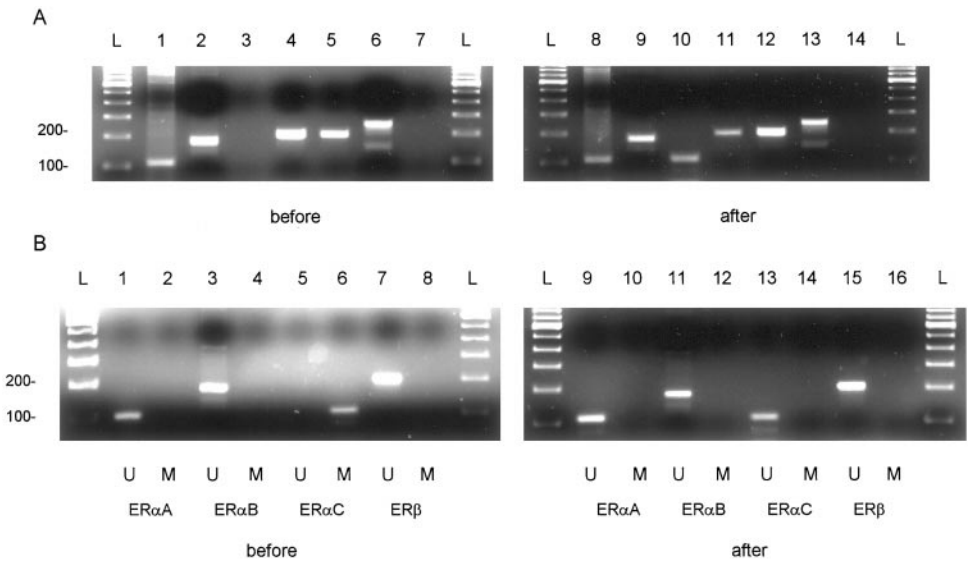

Fig. 2. mRNA expression and methylation status of uterine endometrial cancer cell line, Ishikawa cell line, before and after treatment with the demethylating reagent, 5-azaC. *A*, mRNA expression. The RT-PCR of ERα-A, ERα-B, and ERα-C in uterine endometrial cancer cell line, Ishikawa: *L*, 100-bp ladder marker; *U*, unmethylated bands; *M*, methylated bands. *Lanes 1–7*, RT-PCR using cell line without 5-azaC; *Lanes 8–14*, ERα-A, ERα-B, ERα-C, and ERβ gene expression in cell lines treated with 5-azaC. *Lanes 1* and *8*, expression of ERα-A; *Lanes 2* and *9*, expression of ERα-B; *Lanes 3* and *10*, expression of ERα-C; *Lanes 4* and *11*, ERα expression common to three isoforms; *Lanes 5* and *12*, expression of ERβ; *Lanes 6* and *13*, β-actin mRNA expression as positive controls; *Lanes 7* and *14*, negative controls. *B*, methylation status. *Lanes 1–8*, methylation-specific PCR using cell line without 5-azaC; *9–16*, MSP using cell lines treated with 5-azaC. *Lanes 1*, *2*, *9*, and *10*, unmethylated and methylated bands by ERα-A primers; *Lanes 3*, *4*, *11*, and *12*, unmethylated and methylated bands by ERα-B primers. *Lanes 5*, *6*, *13*, and *14*, unmethylated and methylated bands by ERα-C primers; *Lanes 7*, *8*, *15*, and *16*, unmethylated and methylated bands by ERβ primers.

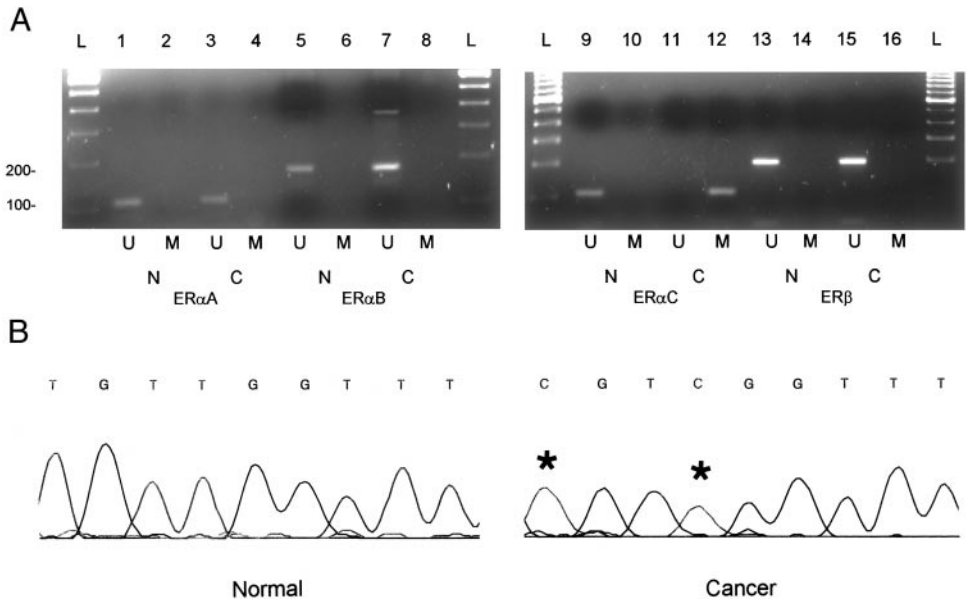

Fig. 3. Methylation status of three ERα isoforms and ERβ in cancerous and normal endometrium tissues: *A, L,* 100-bp ladder marker; *U,* unmethylated bands; *M,* methylated bands; *N,* normal tissues; *C,* cancerous tissues. *Lanes 1–4,* unmethylated and methylated ERα-A alleles; *Lanes 5–8,* unmethylated and methylated ERα-B alleles; *Lanes 9–12,* unmethylated and methylated ERα-C alleles; *Lanes 13–16,* unmethylated and methylated ERβ alleles. *B,* examples of direct DNA sequencing chromatogram for ERα-C. CpG-methylated cytosines remained as cytosines, whereas unmethylated cytosines changed to thymidines in the PCR products. CpG sites are *underlined,* and all cytosines are deaminated and converted to thymines in normal tissue, whereas 5-methylcytosines remain unaltered in cancer tissue (*).

expression in all ERα-C-negative cell lines. Our results clearly demonstrate that there is a tight relationship between the inactivation of ERα-C gene and CpG methylation of ERα-C. CpG-enriched regions are also found in other members of the hormone receptor superfamily, including the androgen, progesterone, and ER genes (1, 2). We have recently observed hypermethylation of the ERα-A in prostate cancer during carcinogenesis (18). We reported that hypermethylation of the ERα-A is associated with ERα-A inactivation in prostate cancer tissues and cultured prostate cancer cell lines (18).

In this study, we also investigated the methylation status of various ERs in 88 cancerous and 46 normal endometrial tissues. ERα-A, ERα-B, and ERβ were unmethylated in cancerous and normal samples. However, we found ERα-C hypermethylation in 94% of cancerous samples, whereas it was unmethylated in all 46 normal samples. We also found ERα-C methylation in 32 pairs of cancerous and normal endometrial tissues from the same patient. ERα-C was methylated in the 91% of cancer tissues, whereas it was unmethylated in all normal endometrial tissues.

ER are key components in the signal transduction pathways controlled by estrogen (1, 2). These pathways direct a variety of physiological processes, such as establishment and maintenance of female sex differentiation patterns, reproductive cycle and pregnancy, and embryonic and fetal development (1, 4, 17). It is also well established that some of these pathways have influence in the carcinogenesis of several cancers (11–14). It is obvious that multiple promoter regions correspond to a variety of controls to the different tissues and cancers (4, 17). Other studies reveal that CpG islands in the 5′-region of the ERα-A and ERα-B are methylated in a significant fraction of primary human breast cancers (19, 20); however, there is no information about ERα isoforms in endometrial cancer. This is the first report on extensive studies of ER and their isoforms in endometrial cancer. Inactivation of ERα-C gene through CpG methylation may be important in pathogenesis of endometrial cancer.

Table 3 *Methylation status of ERs in cancer and normal tissues*

			Cancer tissues		Normal tissues
A.	ERα-A	Unmethylated	88/88	Unmethylated	46/46
		Methylated	0/88	Methylated	0/46
	ERα-B	Unmethylated	88/88	Unmethylated	46/46
		Methylated	0/88	Methylated	0/46
	ERα-C	Unmethylated	5/88	Unmethylated	46/46
		Methylated	83/88	Methylated	0/46
	ERβ	Unmethylated	0/88	Unmethylated	46/46
		Methylated	88/88	Methylated	0/46

	FIGO[a] stage	Unmethylated	Methylated
B.	4b	0/3	3/3
	3c	0/11	11/11
	3a	0/16	16/16
	2b	1/7	6/7
	1c	0/18	18/18
	1b	3/26	23/26
	1a	1/7	6/7

	Pathological Type	Unmethylated	Methylated
C.	Endometrioid	4/67	63/67
	Adenosquamous	0/2	2/2
	Adenoacanthoma	0/5	5/5
	Clear cell	0/2	2/2
	Unknown type	1/12	11/12

[a] FIGO, International Federation of Gynecologists and Obstetricians.

Table 4 *Properties of cancer tissues and normal tissues from the same patient*

	Cancer tissues	Normal tissues
ERα-A		
Unmethylated	32/32	32/32
Methylated	0/32	0/32
ERα-B		
Unmethylated	32/32	32/32
Methylated	0/32	0/32
ERα-C		
Unmethylated	3/32	32/32
Methylated	29/32	0/32
ERβ		
Unmethylated	32/32	32/32
Methylated	0/32	0/32

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