

Loss of estrogen receptor β isoform expression and its correlation with aberrant DNA methylation of the 5'-untranslated region in human epithelial ovarian carcinoma

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Evidence exists that sex steroids such as estrogens affect epithelial ovarian cancer. The expression profiles of the estrogen receptors (ER) and ER β in particular have not been fully described. Therefore, in our present study, we examined the methylation status of the promoters OK and ON, and the expression of ER β isoforms in human epithelial ovarian carcinoma. We then correlated methylation status with ER expression status. Twelve ovarian carcinoma cell lines, six primary cultures of ovarian surface epithelial cells (OSE), and 64 cases of ovarian carcinoma tissues were examined. Bisulfite sequencing and quantitative reverse transcription-polymerase chain reaction were used to evaluate methylation status and expression of ER β isoforms. The relative abundance of exon ON, ER β 1, ER β 2, and ER β 4 mRNA was significantly lower in ovarian cancer cell lines and tissues than in their corresponding normal counterparts. However, ER β 5 mRNA level was relatively higher in the cancers, in clear cell adenocarcinoma in particular, than in the normal ovary. Bisulfite sequencing analysis demonstrated that the two promoters of the ER β gene exhibited distinct methylation patterns. Promoter ON was unmethylated in OSE, rarely methylated in normal ovarian tissues, and extensively methylated in ovarian cancer cell lines and tissues (11/15 cell lines and 18/32 cancer tissues were extensively methylated). The promoter OK was, however, unmethylated in both normal and malignant ovarian cells and tissues. A significant correlation between promoter ON hypermethylation and the loss of exon ON, ER β 1, ER β 2, and ER β 4 mRNA expression was detected in ovarian carcinoma cells and tissues. Treatment of ovarian carcinoma cells with 5-aza-2'-deoxycytidine resulted in reexpression of the ER β gene. The results of our present study suggest that ER β is inactivated mainly through aberrant DNA methylation. This process may play an important role in the pathogenesis of epithelial ovarian cancer. (*Cancer Sci* 2008; 99: 2365-2372)

Of the gynecological malignancies, epithelial ovarian cancer is the leading cause of death in the great majority of developed countries.⁽¹⁾ Sex steroid hormones have been implicated in the etiology and progression of some epithelial ovarian cancers. Human epithelial ovarian carcinomas can express both estrogen receptors (ER) and progesterone receptors (PR).^(2,3) In endometrioid endometrial and breast carcinomas, steroid hormone receptor status is correlated well with response to hormonal manipulation and prognosis.⁽⁴⁻⁶⁾ However, in epithelial ovarian carcinoma, the prognostic significance of tumor ER and PR status remains controversial.^(2,7,8) Forty to 60% of ovarian cancers express ER α , but only a small proportion of patients (7-18%) respond clinically to anti-estrogen treatment.^(9,10)

Most estrogen actions are mediated through its binding to two ER proteins, ER α and ER β , which belong to a large family of

transcription factors, the nuclear receptor family.⁽¹¹⁾ Wild-type ER β (ER β 1) encodes the full-length, 530 amino-acid receptor protein, whereas ER β 2 (ER β cx) to ER β 5, which utilize alternative exons, encode variant receptors with different C- and N-termini.^(7,12) Two novel ER β mRNA isoforms containing distinct 5'-untranslated regions, exons OK and ON, have recently been demonstrated to be generated by alternative splicing of exon 1.⁽¹³⁾ These results indicate that the transcription of the human ER β gene occurs from at least two different promoters, those of ON and OK.

DNA methylation has an essential regulatory function in mammalian development, suppressing gene activity by changing chromatin structure.^(14,15) Aberrant DNA methylation of promoter region CpG islands may therefore serve as an alternate mechanism that leads to the inactivation of tumor-suppressor genes in human cancers.⁽¹⁶⁾ Therefore, the identification of genes regulated by methylation-associated silencing could lead to the characterization of novel regulators of the initiation and progression of human neoplasia.

In the mammary gland, ER β 1 mRNA is downregulated during breast tumorigenesis.⁽¹⁷⁻¹⁹⁾ Decreases in the mRNA expression levels of ER β 1 and ER β 2 in breast carcinoma are significantly correlated with the status of promoter ON hypermethylation.⁽¹⁹⁾ The expression of ER β 1 mRNA is also significantly decreased in localized prostatic adenocarcinoma compared with normal tissues, and this is correlated with aberrant DNA methylation of promoter ON.⁽²⁰⁾ In ovarian carcinoma, several studies have demonstrated that the expression of ER β 1 is decreased in ovarian cancer tissues compared with normal ovarian tissue.⁽²¹⁻²³⁾ ER β 1 functions as an important regulator of cell proliferation and has a proapoptotic role in ovarian cancer cells. This suggests that ER β 1 is a potential tumor-suppressor gene in this tumor.⁽²⁴⁾ However, the expression of the various ER β isoforms in epithelial ovarian cancer has not been described and the mechanism of their action is unclear. Therefore, in the present study, we measured the mRNA expression levels of the ER β isoforms and correlated expression levels with the DNA methylation status of the OK and ON promoters.

Materials and Methods

Surgical specimens and clinical data. Sixty-four specimens of histologically confirmed primary ovarian carcinomas were obtained from patients who underwent surgical therapy from

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Table 1. Characteristics of epithelial ovarian cancer patients in the present study

| Characteristic | n | % |
|----------------|----|------|
| Age | | |
| <50 years | 22 | 34.4 |
| >50 years | 42 | 65.6 |
| Stage | | |
| I/II | 24 | 37.5 |
| III/IV | 40 | 62.5 |
| Histology | | |
| Serous | 31 | 48.4 |
| Endometrioid | 8 | 12.5 |
| Mucinous | 7 | 10.9 |
| Clear cell | 17 | 26.6 |
| Grade | | |
| 1 | 29 | 45.3 |
| 2 | 25 | 39.1 |
| 3 | 10 | 15.6 |

1998 to 2004 at Tohoku University Hospital. Six specimens of normal ovary were also obtained from patients with benign non-ovarian disease. The samples were stored at -80°C and embedded immediately in OCT compound (Sakura Finetechnical, Tokyo, Japan) until further use. Only sections containing a minimum of 80% carcinoma by examination with hematoxylin-eosin staining were used for total RNA preparation. Clinicopathological variables for the patients in this study, including International Federation of Gynecology and Obstetrics (FIGO) stage, histological subtype, and grade, are summarized in Table 1. Histological subtypes and stage were determined according to FIGO criteria. Grade was evaluated by one of the authors (J.A.) using a universal grading system for epithelial ovarian cancer.⁽²⁵⁾ Overall survival was calculated from the time of initial surgery to death, or the date of last contact. The survival times of patients still alive or lost to follow up in September 2007 were censored. The research protocol was approved by the ethics committee of Tohoku University Graduate School of Medicine.

Ovarian cancer cell lines and primary culture of surface epithelial cells. OVCAR3, Caov3, SKOV3, TOV112D, TOV21G, OV90, and ES2 (adenocarcinoma, OVCAR3, SKOV3; serous adenocarcinoma, Caov3, OV90; clear cell adenocarcinoma, TOV21G, ES2; endometrioid adenocarcinoma, TOV112D) cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). JHOS2, JHOS3, JHOS4, HTOA, OMC3, JHOC5, JHOC7, and JHOC8 (serous adenocarcinoma, JHOS2, JHOS3, JHOS4, HTOA; mucinous adenocarcinoma, OMC3; clear cell adenocarcinoma, JHOC5, JHOC7, JHOC8) cell lines were purchased from Riken Cell Bank (Tsukuba, Japan). Cell lines derived from prostate cancer (PC3), breast cancer (MCF-7), and endometrial cancer (Ishikawa cells) were also analyzed.

Primary cultures of normal human ovarian surface epithelium (OSE) cells were initiated from surface scrapings of normal ovaries removed from eight women undergoing abdominal total hysterectomy or radical hysterectomy for non-ovarian disorders at Tohoku University Hospital. The purity of OSE cells was confirmed by immunostaining for cytokeratin and vimentin.⁽²⁶⁾

RNA preparation and real-time quantitative reverse transcription-polymerase chain reaction. Total RNA was isolated from frozen tissues, ovarian cancer cell lines, and OSE cells with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). A reverse transcription-polymerase chain reaction (RT-PCR) kit (Superscript First-Strand Synthesis System; Invitrogen, Carlsbad, CA, US) was used and complementary DNA (cDNA) synthesis was carried out according to the kit instructions.

Real-time quantitative polymerase chain reaction was carried out using the Light Cycler system (Roche Diagnostics, Mannheim, Germany). The primers used were: exon 0K, 5'-TACTGAGTCCGATGAATGTG-3' and 5'-TAAGGCTAGATGGTGAGTTT-3'; exon 0N, 5'-GTCCGCATTTAGAGAAGGC-3' and 5'-GGAGGAAGGAGAATTAAGGCT-3'; ER β 1, 5'-CCTGGCTAACCTCCTGATGC-3' and 5'-ACCCCGTGATGGAGGACTT-3'; ER β 2, 5'-GATCTTGTCTGGACAGGGATG-3' and 5'-AGGCCTTTTCTGCCCTC-3'; ER β 3, 5'-ATGCTTTGGTTTGGGTGAT-3' and 5'-CTTCCCTCAGCATAACAATC-3'; ER β 4, 5'-CTTTGGTTTGGGTGATTG-3' and 5'-CACATGACTCTTTGGCACTA-3'; ER β 5, 5'-CTTTGGTTTGGGTGATTG-3' and 5'-TTCCTTAACCTGCAGACACT-3'; ER α , 5'-AGACACTTTGATCCACCTGA-3' and 5'-CAAGGAATGCGATGAAGTAG-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-ATGGTGGTGAAGACGCCAGT-3'. GAPDH cDNA fragments were amplified as positive controls. Negative controls without RNA and without reverse transcriptase were also done. Two independent RT-PCR reactions were carried out.

DNA preparation and bisulfite sequencing. Genomic DNA from frozen tissues, ovarian cancer cell lines, and OSE cells was extracted using the AquaPure Gnomc DNA kit (Bio-Rad, Hercules, CA, USA). Genomic DNA (1 μg) was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Sequence analysis of genomic DNA templates treated with bisulfite was carried out as described previously.⁽²⁷⁾ The primers used were: exon 0N, 5'-AGATTTTTTAAATTTGAGATTGGGGTTG-3' and 5'-CTTACCTTACAAATAAACACACC-3'; and exon 0K, 5'-GTTGGGGTATTYYGGGGTTGTT-3' and 5'-CCTCCAACAAACACTTCA-3'. The schematics of the primer sequences are depicted in Figure 1. Hot-start polymerase chain reaction was carried out at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C (62°C for exon 0K) for 30 s, and elongation at 72°C for 1 min. The correct-sized band was isolated and its DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen). The purified DNA was sequenced with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis. Statistical analysis was carried out using Stat View 5.0 (SAS Institute Inc., Cary, NC, US) software. The statistical significance among the ER β isoforms and patient characteristics was evaluated using the Mann-Whitney U-test, Kruskal-Wallis analysis of variance, and Scheffe analysis. Univariate analysis of the prognostic significance for prognostic factors was carried out using a log-rank test, after each survival curve was obtained by the Kaplan-Meier method. All patients who could be assessed were included in the intention-to-treat analysis. A result was considered significant when the *P*-value was less than 0.05.

Results

Expression of ER β isoforms in ovarian cancer cells. The gene expression levels of the ER β isoforms in OSE cells and ovarian cancer cell lines are summarized in Figure 2a. Quantitative RT-PCR was carried out and the ratios of the ER β isoforms to GAPDH were calculated to allow for comparison between the cell lines. In OSE cells, the expression pattern for each isoform was relatively homogeneous compared with the heterogeneous pattern in the ovarian cancer cell lines. The median values of exon 0N, ER β 1, and ER β 2 gene expression levels in the cancer cell lines were significantly lower than those in OSE cells ($P < 0.001$) (Fig. 2b). In contrast, the expression of the ER β 5 gene in the cancer cell lines, particularly in the clear cell adenocarcinomas, was significantly higher than in the OSE cells ($P < 0.001$) (Fig. 2a,b). The expression of exon 0K was very

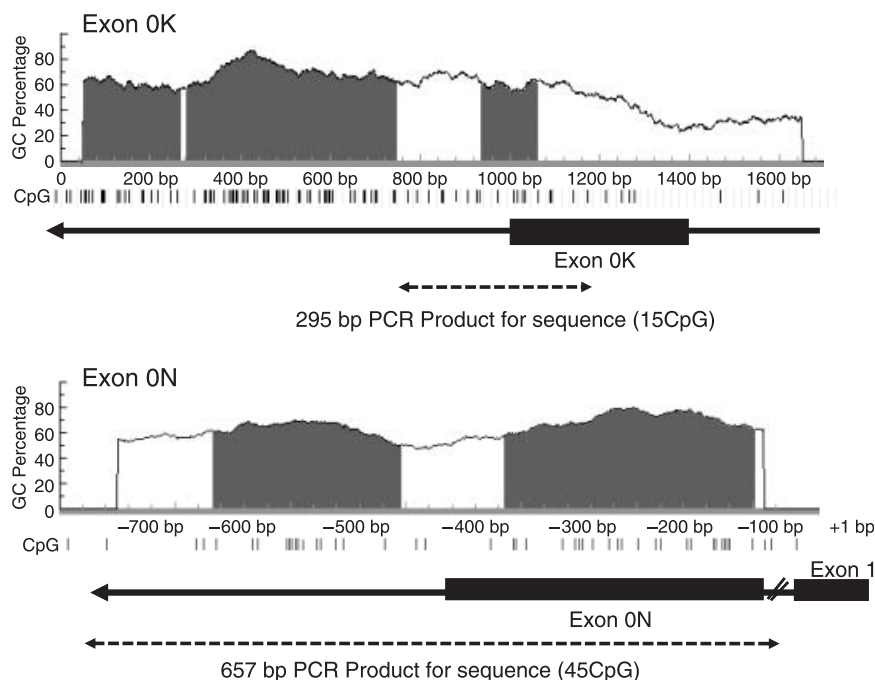


Fig. 1. Schematics of the estrogen receptor- β 5'-flanking region structure, the CpG islands around the promoters of exons 0K and 0N, and the primer sequences for bisulfite sequencing. Vertical lines represent each CpG site. CpG islands were defined as stretches of DNA that had both a >50% GC content and an observed/overexpected frequency of CpG dinucleotides of >0.6.⁽⁴⁰⁾ The polymerase chain reaction (PCR)-amplified region for bisulfite sequencing is indicated by a dashed arrow. The approximately 10-kbp intron between exon 0N and exon 1 is indicated by the diagonal line.

weak in both ovarian carcinoma cell lines and OSE cells (Fig. 2b). The expression patterns in the ovarian cancer cell lines resembled those in cell lines derived from other organs (PC3, MCF7, Ishikawa cells).

Expression of ER β isoforms in ovarian cancer tissues. Gene expression levels of the ER β isoforms in the ovarian cancer tissues are summarized in Figure 3. Median values of all of the ER β isoforms including exon 0N were lower in the ovarian carcinoma tissues than in normal ovarian tissues ($P < 0.001$) (Fig. 3a). In all histological subtypes, the relative amounts of exon 0N and ER β 4 expression were significantly lower in ovarian cancer tissues than in normal ovarian tissues. In serous adenocarcinoma, the expression levels of all ER β isoforms including exon 0N were significantly lower than in normal ovarian tissues ($P < 0.01$) (Fig. 3b). In mucinous and clear cell adenocarcinoma, the expression levels of ER β 1 and ER β 2 were relatively high in comparison to the other histological subtypes, although the differences did not reach statistical significance (Fig. 3b). ER β 5 expression was significantly higher in clear cell adenocarcinoma than in the other histological subtypes, and was comparable to the normal ovarian tissues (Fig. 3b). There were no statistically significant correlations between the expression levels of any of the ER β isoforms or any clinicopathological parameters including prognosis.

Methylation status of the 5'-untranslated region of ER β in ovarian cancer cell lines and tissues. We hypothesized that downregulation of the ER β gene in ovarian cancer occurred through promoter methylation. The comprehensive analysis of the percentage of methylated CpG islands was carried out by bisulfite genomic sequencing (Fig. 4). A 295-bp region of the promoter 0K, containing 15 CpG sites (Fig. 1), was amplified by polymerase chain reaction from bisulfite-modified DNA and the methylation status for the 15 CpG sites was clearly determined by direct sequencing. These results demonstrate that all of the CpG sites were unmethylated in all OSE, ovarian cancer cell lines, and tissues examined in the present study. A 675-bp region of the promoter 0N, containing 45 CpG sites (Fig. 1), was also analyzed. None of five cases and one of six cases were detected to be methylated in the promoter 0N in the OSE and normal ovarian tissues, respectively. Extensive methylation in this region was also detected in most ovarian cancer cell lines and tissues

examined in the present study (11 of 15 cases and 18 of 32 cases, respectively) (Fig. 5). In ovarian carcinoma tissues, the frequency of methylation was higher in clear cell and endometrioid adenocarcinomas (seven of eight cases and four of five cases, respectively) than in serous and mucinous adenocarcinoma (7 of 17 cases and none of two cases, respectively), although the differences were not statistically significant (Fig. 5b).

We further evaluated whether there was any correlation between the mRNA expression levels of the ER β isoforms and the methylation status of promoter 0N. As demonstrated in Figure 5, all of the OSE cells and normal ovarian tissues expressed abundant 0N mRNA, whereas most of the ovarian cancer cell lines showed a loss of, or a very low level of, exon 0N mRNA expression (Fig. 5a). In serous carcinoma tissues, the methylation status of promoter 0N was significantly associated with the loss of exon 0N mRNA expression ($P < 0.05$) (data not shown).

Treatment with 5-aza-2'-deoxycytidine. To further confirm that aberrant DNA methylation contributed to loss of expression of the ER β gene, we assessed the effect of 5-aza-2'-deoxycytidine (5-aza-dC), a demethylating agent, on exon 0N mRNA expression by quantitative RT-PCR. 5-Aza-dC treatment of the ovarian cancer cell lines ES2 and JHOC5, in which the 0N promoter was completely methylated, resulted in marked re-expression of the exon 0N gene on days 3 and 5 (Fig. 6). The 0N mRNA expression after treatment was significantly higher than before treatment ($P < 0.05$) (Fig. 6). In the Caov3 cell line, in which the 0N promoter was completely unmethylated, treatment did not restore expression of 0N mRNA. These results suggest that hypermethylation of the 0N promoter plays a critical role in silencing expression of the ER β isoforms.

Discussion

Aberrant methylation of the ER β promoter 0N was associated with the loss of mRNA expression, which was restored by treatment with the demethylating agent 5-aza-dC. This demonstrates that aberrant DNA methylation is the main pathway of transcriptional silencing of the ER β (0N) gene in ovarian carcinoma cells. In addition, decreased expression of the ER β 1, ER β 2, and ER β 4 genes occurs in a substantial proportion of

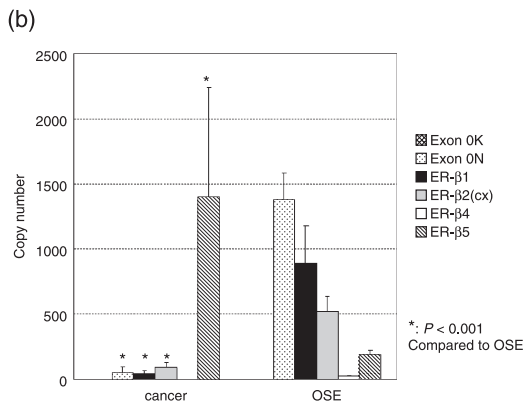
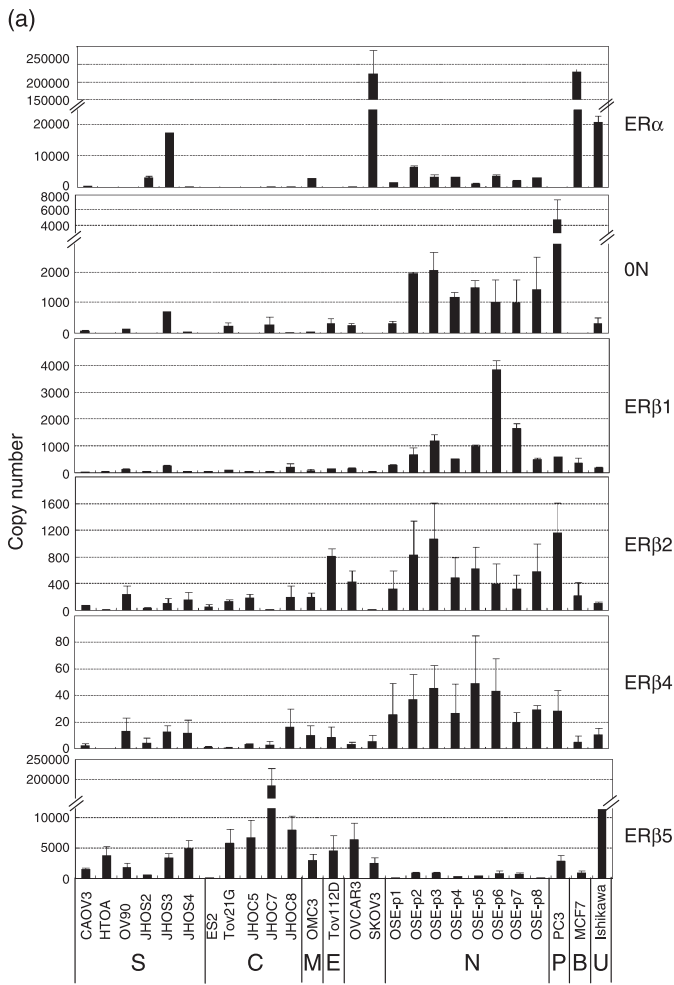


Fig. 2. Expression of estrogen receptor (ER)- β isoform mRNA in ovarian cancer cell lines, normal ovarian surface epithelial cells in primary culture (OSE), PC3, MCF3, and Ishikawa cells. (a) Each bar is the mean \pm SD of two independent quantitative real-time reverse transcription-polymerase chain reaction experiments, standardized against an internal positive control (glyceraldehyde 3-phosphate dehydrogenase). The capital letters at the bottom of the figure refer to the origin of the cell lines (B, breast carcinoma; C, clear cell adenocarcinoma; E, endometrioid adenocarcinoma; M, mucinous adenocarcinoma; N, normal ovarian surface epithelial cells; P, prostatic carcinoma; S, serous adenocarcinoma; U, uterine carcinoma). Summary of the expression levels of ER β isoform mRNA in ovarian cancer cell lines and OSE. (b) Each bar is the median \pm SE of the expression levels of ER β isoforms as shown in (a). Asterisks denote significant differences compared with OSE.

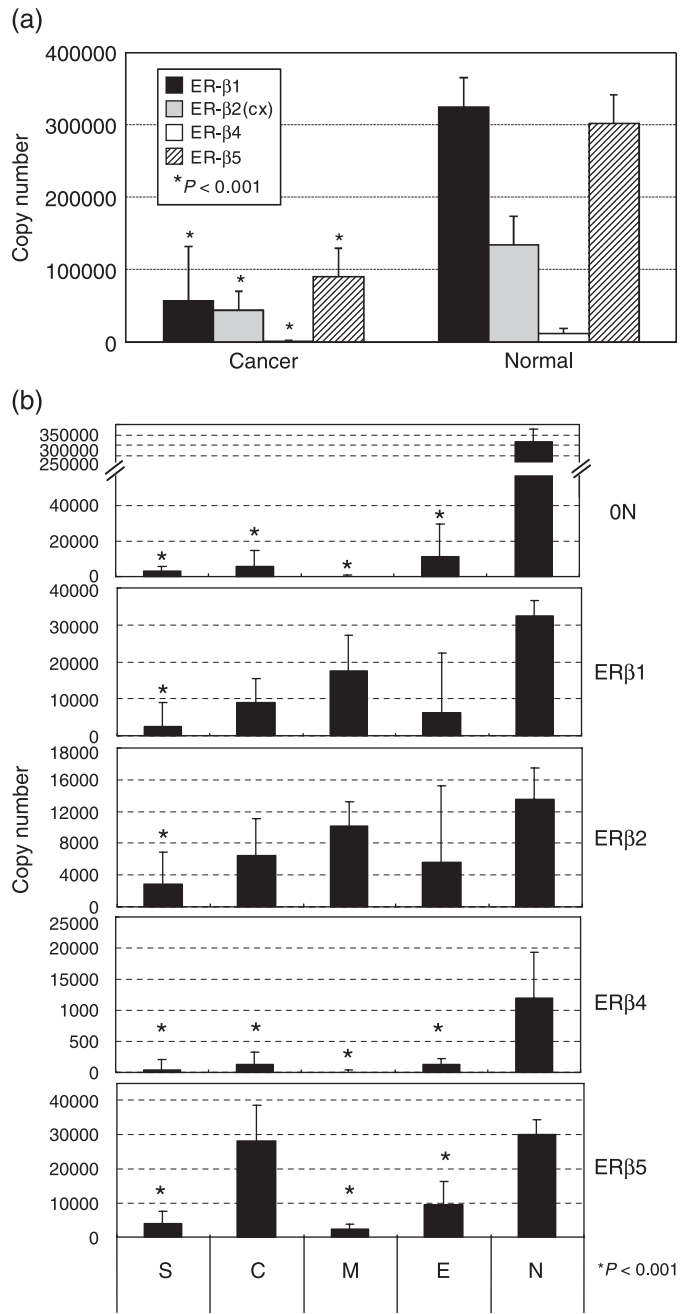
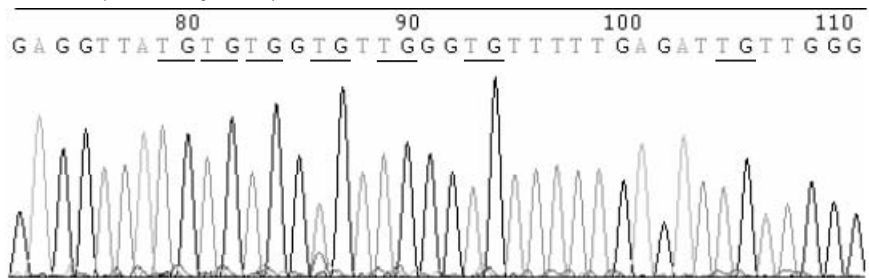


Fig. 3. (a,b) Summary of the expression of estrogen receptor (ER)- β isoform mRNA in normal ovarian and whole ovarian cancer tissues (a), and in each histological subtype (b). Each bar is the median \pm SE of two independent quantitative real-time reverse transcription-polymerase chain reaction experiments for ER β isoforms, standardized against an internal positive control (glyceraldehyde 3-phosphate dehydrogenase; GAPDH). Asterisks denote significant differences ($P < 0.001$) from the normal ovarian tissues. C, clear cell adenocarcinoma; E, endometrioid adenocarcinoma; M, mucinous adenocarcinoma; N, normal ovary; S, serous adenocarcinoma.

ovarian cancer cells and tissues. This suggests that the reduced expression levels of these genes are associated with reduced expression of the ER β (ON) gene. However, ER β 5 was increased in ovarian cancer cells. These results suggest that ER β isoforms may be involved in the development and progression of epithelial ovarian cancer. To the best of our knowledge, this is the first report describing epigenetic silencing of ER β isoforms in human ovarian cancer.

Caov-3 (Unmethylated)



SKOV-3 (Methylated)

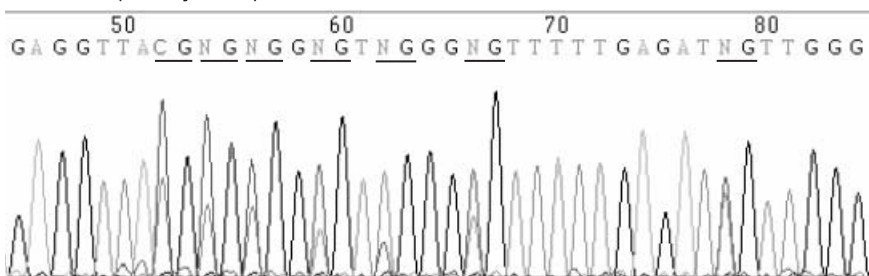


Fig. 4. Representative DNA sequences of bisulfite-treated DNA from ovarian cancer cell lines. CpG sites are underlined. Genomic DNA was modified with sodium bisulfite. This treatment resulted in the conversion of unmethylated cytosines to thymines, whereas the methylated cytosines remained unchanged. The degree of methylation at each CpG site was expressed as the peak height of the methylated cytosine relative to the peak height of total cytosine. No methylation of the promoter 0N region was present in the Caov-3 cell line and partial methylation was present in the SKOV-3 cell line.

Changes in the mRNA and protein levels of the ER β isoforms have been documented in various cancerous tissues. Campbell-Thompson *et al.* reported that ER β is the predominant ER subtype in the human colon and that decreased levels of ER β 1 and ER β 2 mRNA is associated with colonic tumorigenesis in women.⁽²⁸⁾ Leygue *et al.* reported that the expression levels of ER β 2 and ER β 5 mRNA are higher than that of ER β 1 mRNA in cancer cell lines and breast tumors, and that changes in the relative expression of ER β 1, ER β 2, and ER β 5 mRNA occur during breast tumorigenesis and tumor progression.⁽²⁹⁾ Zhao *et al.* demonstrated that both ER β 1 and ER β 2 mRNA levels are significantly lower in tumor cell lines than in normal breast epithelial cells, although ER β 2 mRNA expression is more pronounced than that of ER β 1.⁽¹⁹⁾

In prostate cancer, higher expression of ER β 2 is associated with poor survival.⁽³⁰⁾ Leung *et al.* reported that levels of ER β 1 expression are low when compared with those of other ER β isoforms in various cell lines and tissues.⁽³¹⁾ Skrzypczak *et al.* noted that some adenocarcinomas show a slight decrease in ER β 2 mRNA and a significant increase in ER β 5 mRNA.⁽³²⁾ In epithelial ovarian cancer, several previous studies have demonstrated that ER β mRNA levels are decreased in ovarian cancer samples compared to normal ovaries, whereas the level of ER α mRNA is similar or slightly higher in cancer samples compared to normal ovaries.^(21–24) ER β 2 (originally referred to as ER β cx) is expressed in several ovarian epithelial tumor types, whereas its splice variant (originally referred to as ER β 2) and the exon 5/6 deletion variant are expressed only at very low levels.⁽³³⁾ However, detailed descriptions of the expression levels of the different ER β isoforms in ovarian cancer have not been published, likely because previous studies have had small sample sizes with few normal samples for comparison.

The present study utilized substantial numbers of ovarian cancer cell lines and tissues in order to make valid correlations between the various ER β isoforms and clinicopathological parameters including the histological subtype. Additionally, primary cultures of OSE were used as a normal control to reduce the contamination from stromal components. We studied the expression of those ER β isoforms with a truncation, insertion, or exchange of sequences in the C-terminal of ligand-binding domains (ER β 1, ER β 2, ER β 4, ER β 5) as they were the most frequently observed ER β isoforms in the breast, endometrial,

prostate, and colon cancers described above. We did not investigate the expression of ER β 3 because ER β 3 expression appears to be restricted to the testis.⁽⁷⁾

The expression levels of ER β 1, ER β 2, and ER β 4 mRNA were decreased in both ovarian cancer cell lines and cancer tissues in comparison to normal ovarian tissues. The results, especially those for ER β 1 and ER β 2, are consistent with previous studies in that their expression levels are decreased in ovarian cancer. Bardin *et al.* reported that ER β expression is decreased in cysts and ovarian carcinomas in comparison to normal ovaries. They demonstrated that ER β strongly inhibits PEO14- and BG1-dependent cell proliferation and cell motility in a ligand-independent manner, and induces apoptosis when transfected into cells.⁽²⁴⁾ Omoto *et al.* demonstrated that MCF-7 cells stably expressing ER β 1 or ER β 2 display reduced cell proliferation and colony formation in anchorage-independent conditions.⁽³⁴⁾ Additionally, expression of ER β in ER α -positive breast cancer cells inhibits their growth.⁽³⁵⁾ Interestingly, ER β knockout analysis has shown that ER β is critical for the regulation of epithelial growth, and its absence results in hyperplasia of the prostatic epithelium.⁽³⁶⁾ Taken together, these data clearly demonstrate that ER β is an inhibitor of proliferation and that the loss of ER β expression could be an important event in the pathogenesis of estrogen-dependent cancers. It is possible that ER β acts as a tumor-suppressor gene. If this is the case, our data suggest that ER β 1 is more important as a tumor suppressor in ovarian cancer because ER β 1 is more comprehensively repressed in ovarian cancers compared with other ER β isoforms. The identification of ER β -regulated specific genes involved in epithelial proliferation and apoptosis may advance our understanding of the progression of ovarian cancer and aid in the design of new targeted therapies.

In contrast to ER β 1, ER β 2, and ER β 4, the expression of ER β 5 mRNA was increased in ovarian cancer cell lines compared to OSE. Remarkably, ER β 5 was strongly expressed preferentially in clear cell adenocarcinoma cell lines and tissues. Clear cell adenocarcinoma has a poor prognosis, is relatively resistant to conventional chemotherapy, and has a characteristic molecular profile.^(3,37) The ER β 5 mRNA expression level in human breast tumors and endometrial tumors exceeds that of ER β 1. Functionally, ER β 5 heterodimerizes with ER β 1 and significantly enhances its overall activity in a ligand-dependent manner.⁽³¹⁾ Even though little is currently known about ER β 5, we suspect

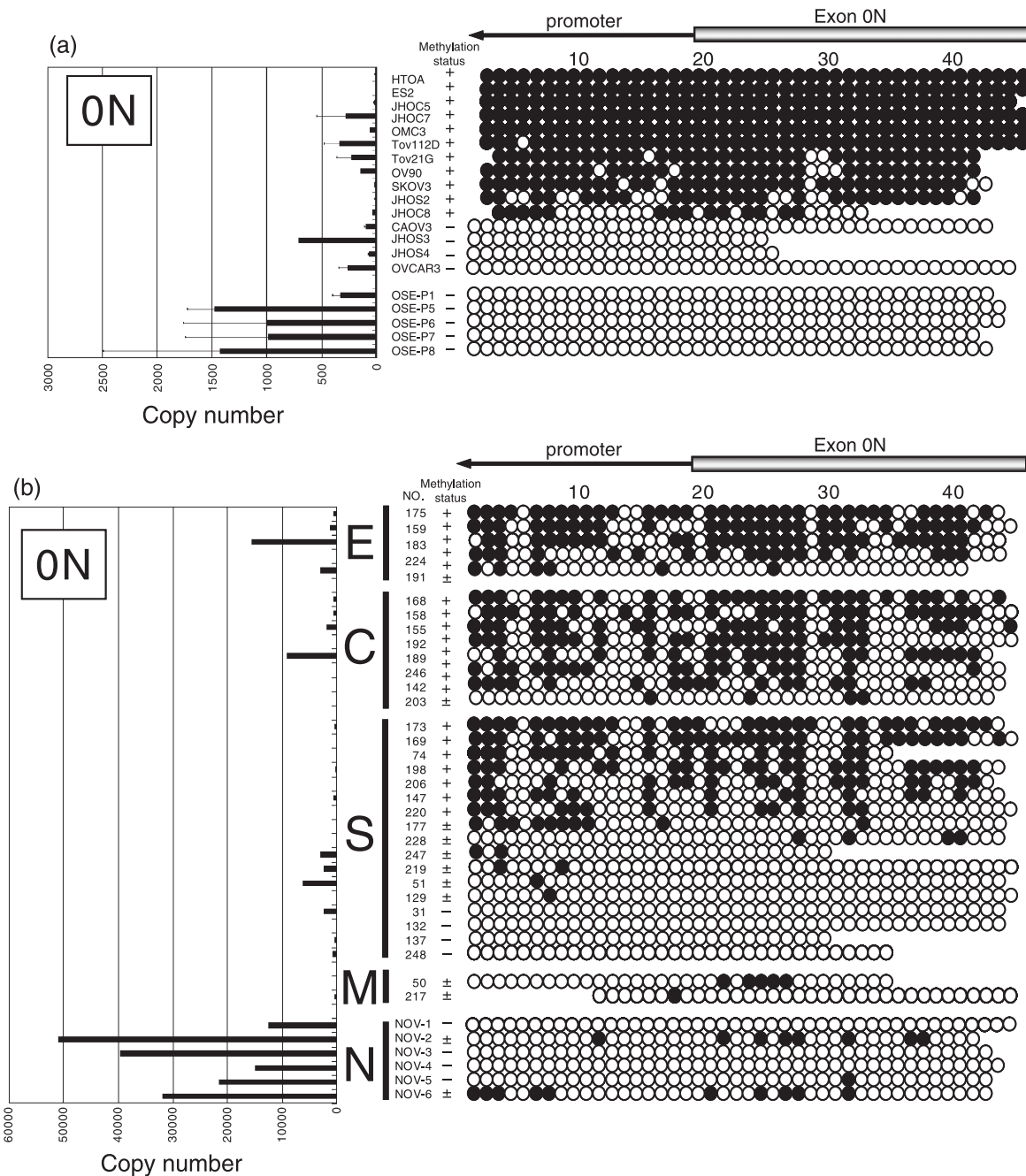


Fig. 5. Methylation status of each CpG site in the 0N promoter and expression of 0N mRNA in cells and tissues. Genomic DNA was extracted from 15 ovarian cancer cell lines, five normal ovarian surface epithelial cells in primary culture (OSE), 32 primary ovarian cancer tissues, and six normal ovarian tissues. (a) Ovarian cancer cell lines and OSE. (b) Ovarian cancer and normal ovarian tissues. After bisulfite treatment, direct sequencing was carried out for 45 CpG sites at the exon 0N promoter. Each circle represents a CpG site, a filled circle indicates full or partial methylation, and an open circle indicates no methylation. Methylation status indicated extensive methylation (30–100% methylation of promoter 0N CpG sites, +), marginal methylation (1–29% methylation of promoter 0N CpG sites, ±), and unmethylation (0% methylation of promoter 0N CpG sites, -). Relative expression levels of exon 0N mRNA are shown on the left side. C, clear cell adenocarcinoma; E, endometrioid adenocarcinoma; M, mucinous adenocarcinoma; N, normal ovary; S, serous adenocarcinoma.

that the effects of either estrogens or antiestrogens may be mediated through dimerization of ERβ1 and ERβ5. This isoform may influence cellular proliferation in ovarian cancer, especially in the clear cell subtype.

With regard to the mechanism behind ERβ gene repression, no mutations or loss of heterozygosity at this gene locus have been reported in ovarian cancer.⁽³⁸⁾ Recent studies have, however, demonstrated methylation of the ERβ promoter 0N and reduced expression of ERβ isoforms in primary prostate cancer and

breast cancer tissues as well as in cancer cell lines.^(19,20) In the present study, a clear correlation between aberrant methylation and silencing of ERβ 0N mRNA was observed in ovarian cancer cell lines and tissues. Expression was restored by treatment with the demethylating agent 5-aza-dC. These results indicate that hypermethylation of the 0N promoter plays a role in silencing ERβ gene expression. We also observed that the two promoter regions of the ERβ gene, 0N and 0K, exhibited distinct methylation and expression patterns. The 0K promoter was

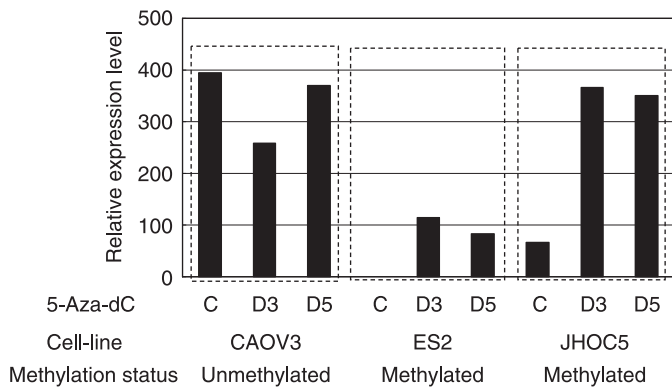


Fig. 6. Reexpression of estrogen receptor (ER)- β ON mRNA by 5-aza-2'-deoxycytidine (5-aza-dC) treatment. Methylation status and mRNA expression of exon ON were assessed by bisulfite sequencing and quantitative reverse transcription-polymerase chain reaction of D0, D3, and D5 with or without 5-aza-dC (1.0 μ mol/L) treatment. Methylation status showed methylation of ON promoter CpG sites of each cell line before 5-aza-dC treatment.

fully unmethylated and repressed in ovarian cancers and normal ovaries. Zhao *et al.* reported that the ON promoter was unmethylated in normal breast epithelial cells, but extensively methylated in breast cancer cell lines. In contrast, the OK promoter was

unmethylated in both normal and malignant breast epithelial cells.⁽¹⁹⁾ Our results are consistent with observations in breast cancer. There is little data regarding the relative contributions of the ON and OK promoters to the final ER β 1 and ER β 2 transcripts. The ER β 1 cDNA sequence does contain exon ON, whereas ER β 2 cDNA sequences contain either exon ON or exon OK.^(7,12) Leung *et al.* reported that ER β isoforms including ER β 1, ER β 2, and ER β 4, but not ER β 5, were found to be under the control of both the OK and ON promoters, whereas ER β 5 was regulated exclusively by the OK promoter in breast cancer.⁽³⁹⁾ Further studies characterizing the OK and ON promoters as well as putative promoters in other locations are needed to completely elucidate the entire mechanism regulating human ER β gene expression. Aberrant CpG island methylation is an epigenetic change that is largely responsible for the silencing of ER β isoforms. Further studies are needed to clarify the potential role of ER β as a tumor suppressor in the pathogenesis of ovarian cancer.

Acknowledgments

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