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# Single CpG site methylation controls estrogen receptor gene transcription and correlates with hormone therapy resistance

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ABSTRACT

Hormone therapy is the most effective treatment for patients with estrogen receptor α-positive breast cancers. However, although resistance occurs during treatment in some cases and often reflects changed estrogen receptor α status, the relationship between changes in estrogen receptor α expression and resistance to therapy are poorly understood. In this study, we identified a mechanism for altered estrogen receptor α expression during disease progression and acquired hormone therapy resistance in aromatase inhibitor-resistant breast cancer cell lines. Subsequently, we investigated promoter switching and DNA methylation status of the estrogen receptor α promoter, and found marked changes of methylation at a single CpG site (CpG4) in resistant cells. In addition, luciferase reporter assays showed reduced transcriptional activity from this methylated CpG site. This CpG region was also completely conserved among species, suggesting that it acts as a methylation-sensitive Ets-2 transcription factor binding site, as confirmed using chromatin immunoprecipitation assays. In estrogen receptor α-positive tumors, CpG4 methylation levels were inversely correlated with estrogen receptor α expression status, suggesting that single CpG site plays an important role in the regulation of estrogen receptor α transcription.

## 1. Introduction

Estrogen and estrogen receptor α (ERα) play critical roles in breast cancer development and progression [1,2]. As a member of the nuclear receptor superfamily, ERα functions as a ligand-activated transcription factor and regulates target gene expression [3]. Approximately 70% of breast cancers express ERα and most respond to hormone therapies, such as anti-estrogens (AE) and aromatase inhibitors (AI) [4–6], which have been associated with fewer adverse reactions, and are widely used to treat ERα-positive breast cancers. However, ERα-negative cancers fail to respond to hormone therapies and most are more clinically aggressive, resulting in poor prognoses [7]. Additionally, some ERα-positive cancers acquire resistance during long-term treatment [8,9], often in conjunction with changed ERα expression status [10,11]. Hence, reduced ERα expression may be central to the acquisition of resistance to hormone therapy.

According to previous reports, the ERα gene has at least seven functional promoters [12–17], and investigations of the biological and clinical significance of multiple promoters [15,18–23] indicate tissue and cell type specificity [15,19,21]. Moreover, the presence of multiple promoters on the ERα gene may be associated with varying tumor cell characteristics.

Previous studies suggest that reduced ERα expression in breast cancers follows epigenetic changes such as promoter hypermethylation and histone deacetylation [24–28]. For example, treatment with DNA methyltransferase (DNMT) and/or histone deacetylase (HDAC) inhibitors restores ERα mRNA and functional protein expression [25–28], reflecting restored effects of various transcription factors [29–31]. However, in contrast with primary ERα-negative breast cancers, mechanisms of acquired ERα silencing, resistance to hormone therapy,

*Abbreviations:* AI, aromatase inhibitor; AE, anti-estrogen; DNMT, DNA methyltransferase; ERα, estrogen receptor α; ERE, estrogen-response element; EBS, Ets binding site; GFP, green fluorescent protein; HDAC, histone deacetylase; PgR, progesterone receptor

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and cancer progression remain unknown.

Previously, we established several breast cancer cell lines that were resistant to hormone therapy [32–34]. In particular, two AI resistant model cell lines were simultaneously obtained from the same parental MCF-7 ERα-positive breast cancer cell line under conditions of long-term estrogen depletion, and whereas one of these had increased ERα expression levels, the other had decreased levels [32]. Accordingly, the former cell line was sensitive to AE but not to AI, whereas the latter was resistant to both drugs [32], providing a useful model of altered ERα expression mechanisms for investigations of AI resistant breast cancers.

In this study, mechanisms of altered ERα expression and acquired resistance to AI during cancer progression were analyzed according to ERα gene promoter usage and methylation in established cell lines, and data were compared to those from parental MCF-7 cells. The ensuing comparisons indicate that a single CpG site methylation within a CpG island shore of the ERα gene contributed to alterations in ERα expression and subsequent acquisition of treatment resistance.

## 2. Materials and methods

### 2.1. Cell lines and culture

Estrogen deprivation-resistant (EDR) cells (A1/A2, type 1 V1/V2 and A4/C7, type 2 V1/V2 cells) were established from MCF-7 cells as described previously [32,35]. Briefly, MCF-7 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal calf serum (FCS; Tissue Culture Biologicals, Tulare, CA, USA) and 1% penicillin/streptomycin (Gibco BRL, Grand Island, NY, USA). Type 1 and 2 cells were cultured in phenol red-free RPMI 1640 medium (Gibco BRL) supplemented with 5% dextran-coated charcoal-treated FCS and 1% penicillin/streptomycin. All cells were incubated at 37 °C in an atmosphere containing 5% $CO_2$.

### 2.2. Clinical samples

Primary human breast cancer samples (Table 1) were surgically removed from patients at the Saitama Cancer Center Hospital (Saitama, Japan) between 1999 and 2001 with approval from the Saitama Cancer Center Ethics Committee. Samples were stored after embedding in optimal cutting temperature (OCT) compound and were transferred to Tohoku University. Experimental protocols were approved by the Tohoku University Ethics Committee (Tohoku University No. 2009-81), and were performed in accordance with the Declaration of Helsinki. ERα and progesterone receptor (PgR) statuses were determined using enzyme immunoassays. ER- and PgR-positive status were defined as ≥10 U. DNA was extracted after removal of non-tumor tissues using laser capture microdissection, and methylation statuses were determined using combined bisulfite restriction analyses.

**Table 1**
Patient clinicopathological data.

| Characteristic | | n | Characteristic | | n |
|---|---|---|---|---|---|
| Age | < 50 | 9 | stage | I | 2 |
| | ≥50 | 27 | | II | 22 |
| | | | | III | 6 |
| ER | positive | 17 | | IV | 6 |
| | negative | 19 | | | |
| | | | T | 1 | 4 |
| PgR | positive | 14 | | 2 | 22 |
| | negative | 22 | | 3 | 5 |
| | | | | 4 | 5 |
| Node metastasis | positive | 17 | | | |
| | negative | 19 | N | 0 | 6 |
| | | | | 1 | 24 |
| Tumor size | < 2.5 cm | 13 | | 2 | 3 |
| | ≥2.5 cm | 23 | | 3 | 3 |
| | | | M | 0 | 30 |
| | | | | 1 | 6 |

### 2.3. Quantitative real-time reverse transcription polymerase chain reaction

Transcription products from various ERα gene promoters were measured using quantitative real-time reverse transcription polymerase chain reaction as described previously [23]. Briefly, total RNA was extracted using IsoGen lysis buffer (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's instructions. Extracted RNA (1 μg) was converted to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Transcripts were detected using TaqMan probes with a Step One™ Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). Relative copy numbers were calculated from a standard curve and were normalized to β-actin. Sequences of primers and probes are listed in Supplementary Table S1.

### 2.4. Sodium bisulfite genomic sequencing

Genomic DNA was extracted using a DNA Mini Kit (Qiagen) and was then subjected to sodium bisulfite treatment using a EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Converted DNA was then amplified using bisulfite-specific PCR to obtain products for sequencing. PCR products were then gel-purified using a QIAquick Gel Extraction Kit (Qiagen), and direct sequencing was performed using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Inc.) and a 3500xL Genetic Analyzer (Applied Biosystems, Inc.), and DNA methylation levels were quantified using colony sequencing. Briefly, purified products were inserted into T-easy vectors (Promega Corporation, Madison, WI, USA) and were transformed into competent JM109 cells (TaKaRa Bio Inc., Otsu, Japan). Colonies were then selected on lysogeny broth–ampicillin (100 μg/μl) agar using the IPTG/X-gal method, and plasmid DNA was subsequently extracted using a QIAprep Spin Miniprep kit (Qiagen). Finally, sequencing was performed as described above using the PCR and sequencing primers listed in Supplementary Table S2.

### 2.5. Combined bisulfite restriction analysis (COBRA)

Bisulfite converted fragments of ERα promoters were digested by *BspT104I* (TaKaRa Bio. Inc.) at 37 °C for 4 h, were separated on 2% agarose gels, were stained with SYBR Green, and were then visualized under UV irradiation. Bands were quantified using ImageJ software.

### 2.6. Reporter plasmid construction and luciferase assays

ERα promoter fragments (ERα pro 1.9 kbp: −1757 − + 166; and ERα pro 2.3 kbp: −2138 − + 166) were PCR-amplified using primers with *Bgl*II sites. After digestion with *Bgl*II, fragments were ligated into the pGL3-basic luciferase reporter vector (Promega Corporation). Synthetic oligonucleotides (CpG4 and CpG4 m) were ligated into the *Nhe*I–*Bgl*II sites of the pGL3-promoter vector. All DNA products were confirmed by sequencing. Transient transfection of reporter plasmids was performed using Trans IT LT-1 Transfection Reagent (Mirus Bio LLC, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cancer cells were grown to approximately 50% confluence in 6-cm culture dishes. Reporter plasmids (0.5 μg) were mixed with transfection reagent in serum-free medium and were added to culture medium. The vector pRL-TK (Promega Corporation) was also mixed with transfection reagent (internal transfection efficiency control), and after 24-h incubation, cells were lysed and luciferase activities were determined using a Dual-Luciferase Reporter Assay System (Promega Corporation). PCR primer and synthetic oligonucleotide sequences are