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Clinical implication of estrogen-related receptor (ERR) expression in uterine endometrial cancers

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ABSTRACT

Estrogen receptor (ER)α and ERβ mRNAs levels decreased with clinical stage, myometrial invasion and dedifferentiation. On the other hand, ERRα mRNA levels and histoscores increased with clinical stage and myometrial invasion, regardless of dedifferentiation. ERRα can bind to the steroid receptor coactivator family without any ligands, and drive transcription activity of the target genes. The competitive interaction of ERRα/ER expression associated with the use of common cofactors during loosing estrogen dependency might cause their expression manner. The up-regulation of ERRα might be related to tumor growth and advancement in uterine endometrial cancers. It is speculated that ERRα is a candidate for prognostic factors in uterine endometrial cancer, although ERRs are not directly related to growth of uterine endometrial cancer.

1. Introduction

Uterine endometrial cancer has been recognized as being estrogen-dependent in tumor growth and advancement. However, estrogen receptor (ER) expression in uterine endometrial cancers is decreasing with tumor progression, and estrogen dependency related to growth is lost during advancement. Although estrogens are not ligands for estrogen-related receptor (ERR), one type of orphan receptor, ERR may interact with ER in competition to use the common cofactors for transcription [1–6]. ERRα and β were cloned using the DNA-binding domain of the human estrogen receptor α cDNA as a hybridization probe [7]. ERRγ was identified by yeast two-hybrid screening, using transcriptional coactivator glucocorticoid receptor interacting protein 1 [8]. Distribution and function about ERRs have been limitedly known mainly in mice. ERRα was expressed in various organs during the early embryonic development of the mouse [4,7,9]. In adult mice, ERRα was mainly expressed in kidney, heart, and brown adipocytes and tissues which preferentially metabolize fatty acids. ERRα can control the expression of medium-chain acyl coenzyme A dehydrogenase (MCAD), a key enzyme involved in the mitochondrial beta-oxidation of fat, through the MCAD nuclear receptor response element 1 and thus may play an important role in regulating cellular energy balance in vivo [4]. ERRβ was expressed exclusively during a narrow developmental window in trophoblast progenitor cells between 6.5 and 7.7 days post-coitum [10]. Furthermore, homozygous ERRβ knockout embryos, which have severely impaired placental formation, die at 10.5 days post-coitum, and therefore, ERRβ might have an important role in the early placentation required for normal trophoblast proliferation and differentiation [11]. ERRγ transcripts were abundantly expressed in the isocortex, the olfactory system, cranial nerve nuclei and major parts of the coordination centers, e.g. reticular formation, major parts of the extrapyramidal motor systems and trigeminal ganglion neurons, and thus the distribution of ERRγ was clearly distinguished from that of ERRα, ERRβ and ER, pointing at functional differences between ERRγ and these receptors [12]. Orphan receptor ERRs may interact with ERs in competition to use the common ER cofactors for transcription. Therefore, we studied the manner of novel orphan receptor ERR and ER expressions to know the mechanism of estrogen-related growth in uterine endometrial cancers.

2. Materials and methods

2.1. Patients

Prior, informed consent for the following studies was obtained from all patients and the Research Committee for Human Subjects, Gifu University School of Medicine. Ninety patients ranging from 31 to 86 years of age with uterine endometrial cancers (stage I, 48 cases; stage II, 20 cases; and stage III, 22 cases, and well-differentiated endometrioid adenocarcinoma of the endometrium [G1], 33 cases; moderately differentiated endometrioid adenocarcinoma [G2], 30 cases; and poorly differentiated endometrioid adenocarcinoma [G3], 27 cases) underwent surgery and 20 patients ranging from 35 to 46 years of age with a regular menstrual cycle

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underwent hysterectomy for uterine leiomyoma with histologically normal uterine endometrium at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, between January 2003 and January 2006. None of the patients had received any therapy, including hormonal, before uterine endometrial tissue was taken. The clinical stage of uterine endometrial cancers was determined by International Federation of Obstetrics and Gynecology (FIGO) classification [13]. A part of each tissue was snap-frozen in liquid nitrogen to determine ER and ERR mRNAs.

2.2. Real-time RT-PCR to amplify ERR and ER mRNAs

Total RNA was extracted from tissue specimens using the acid–phenol guanidinium method (ISOGNE), Nippon Gene, Tokyo, Japan). The total RNA (3 μg) was reverse transcribed in 20 μl volume for 1 h at 37 °C with a mixture of 200 units of Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, Gibco BRL) and the following reagents: 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 40 units of RNAsin (Toyobo, Osaka, Japan), 10 mM dithiothreitol (DTT), 0.5 mM deoxyribonucleoside triphosphates (dNTPs) and 30 pmol 3′-end specific primer as detailed below. The reaction was incubated for 5 min at 94 °C to inactivate MMLV-RTase. PCR was constructed in a final volume of 25 μl containing PCR buffer (2 mM Tris–HCl, pH 8.0, 10 mM KCl, 10 μM ethylenediamine tetraacetic acid, 0.1 mM DTT, 0.05% Tween 20, 0.05% Nonidet P-40, 5% glycerol, 3 mM $MgCl_2$), SYBR Green I (1:30,000 dilution, BioWhittaker Molecular Applications, Rockland, ME, USA), 0.3 mM dNTPs, 0.3 μM each of PCR primers and 1.25 units of Takara Ex TaqTM R-PCR Version (Takara Suzo, Otsu, Japan) PCR with the reverse transcribed RNAs as a sample template or a dilution series of the DNA fragment involving the target gene as a standard control. The mixture was amplified for 45 cycles of PCR at 94 °C for 10 s for denaturing, 55 °C for 5 s for annealing and 72 °C for 20 s for extension in a Smart Cycler (Cepheid, Sunnyvale, CA, USA). The PCR primers were as follows:

ERα-S: 5′-CGA GAG GAG TAT GTT CTA CT-3′,
ERα-AS: 5′-TGC AGA GCT TCT CGC AGC T-3′;
ERβ-S: 5′-CCG AGA GCT TGT GGT CAT CA-3′,
ERβ-AS: 5′-ACA CCA GCT TGT CGT CAT AG-3′;
ERγ-S: 5′-TAA TGC TAT CCT GCA GCT GG-3′,
ERγ-AS: 5′-CTG CAG CGC TTC ATG TAA GA-3′;
ERα-S: 5′-TGCTTCAGGCTACCATTATG-3′,
ERα-AS: 5′-CTTTCATCATTCCCACTTCG-3′;
ERβ-S: 5′-TACGCATCGGGATATCACTA-3′,
ERβ-AS: 5′-TCGTAACACTTCCGAAGTCG-3′.

2.3. Immunohistochemistry

Four-micrometer sections of formalin-fixed paraffin-embedded tissues of uterine cervical cancers were cut with a microtome and dried overnight at 37 °C on a silanized-slide (Dako, Carpinteria, CA, USA). Samples were deparaffinized in xylene at room temperature for 80 min and washed with a graded ethanol/water mixture and then with distilled water. The samples were soaked in a citrate buffer, and then microwaved at 100 °C for 10 min. The protocol for a DakoCytomation LSAB+ System-HRP (Dako) was followed for each sample. In the described procedures, mouse anti-human ERRα (Perseus Proteomics, Tokyo, Japan) was used at dilutions of 1:125 as the first antibody. For the negative control of ERRα, the pre-immune mouse serum (Dako) was used instead of the primary antibody.

2.4. Assessment of histochemical score (histoscore)

All sections of immunohistochemical staining for ERRα were evaluated in a semiquantitative fashion according to the method described by McCarty et al. [14], which considers both the intensity and the percentage of cells stained at each intensity. Intensities were classified as 0 (no staining), 1 (weak staining), 2 (distinct staining), 3 (strong staining) and 4 (very strong staining). For each stained section, a value-designated histoscore was obtained by application of the following algorithm: histoscore = $\sum(i+1) \times \mathrm{Pi}$, where i and Pi represent intensity and percentage of cells that stain at each intensity, respectively, and the corresponding histoscores were calculated separately.

2.5. Statistics

ER and ERR mRNA levels and ERRα histoscores were measured from three parts of the same tissue in triplicate. Statistical analysis was performed with Student's t-test. Differences were considered significant when p was <0.05.

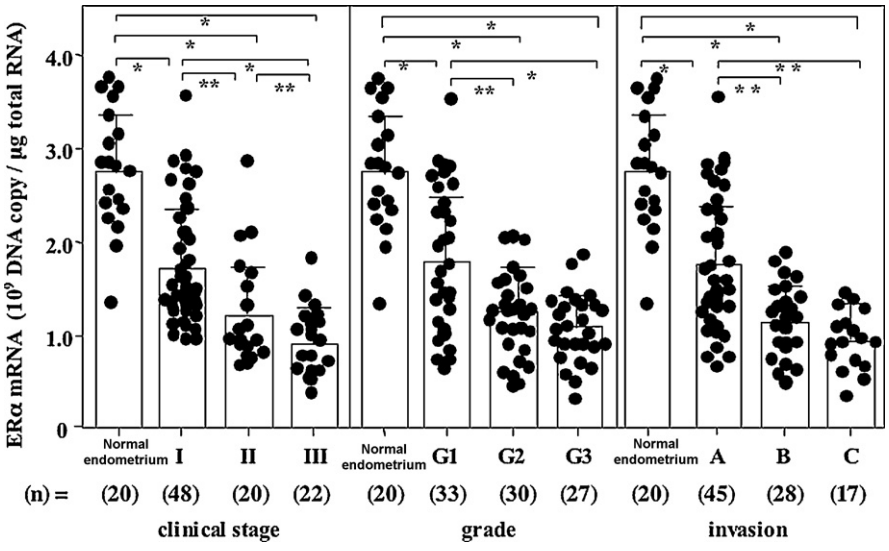

Fig. 1. Levels of ERα mRNA in normal uterine endometria and uterine endometrial cancers classified according to clinical stages, histopathological types and myometrial invasion. G1, well-differentiated endometrioid adenocarcinoma of the endometrium, G2, moderately differentiated endometrioid adenocarcinoma of the endometrium, G3, poorly differentiated endometrioid adenocarcinoma of the endometrium, A, tumor limited to endometrium, B, tumor invades up to less than one half of myometrium, C, tumor invades to more than one half of myometrium. Each level is the mean of 9 determinations. *$p < 0.001$; **$p < 0.05$.

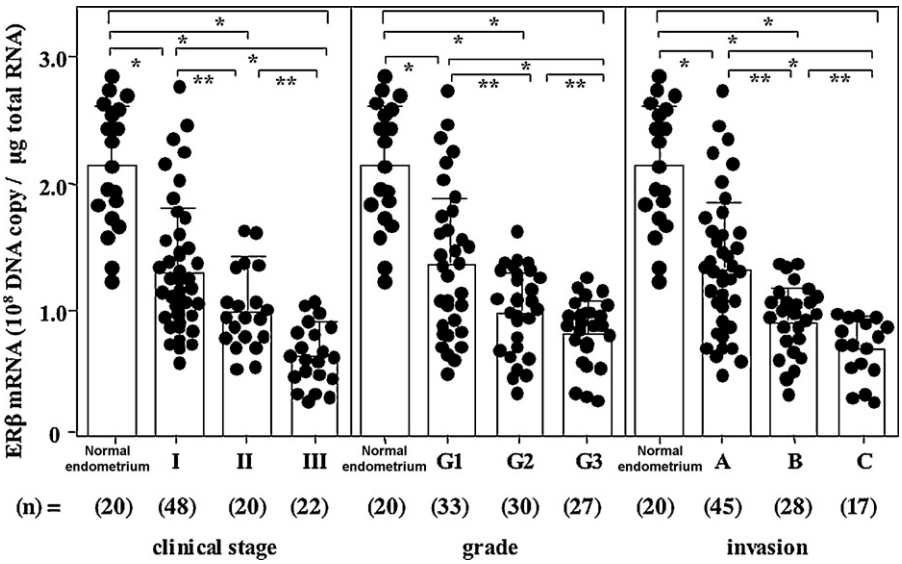

Fig. 2. Levels of ERβ mRNA in normal uterine endometria and uterine endometrial cancers classified according to clinical stages, histopathological types and myometrial invasion. Abbreviations of histopathological type and myometrial invasion were followed as in Fig. 1. Each level is the mean of 9 determinations. $^{*}p < 0.001$; $^{**}p < 0.05$.

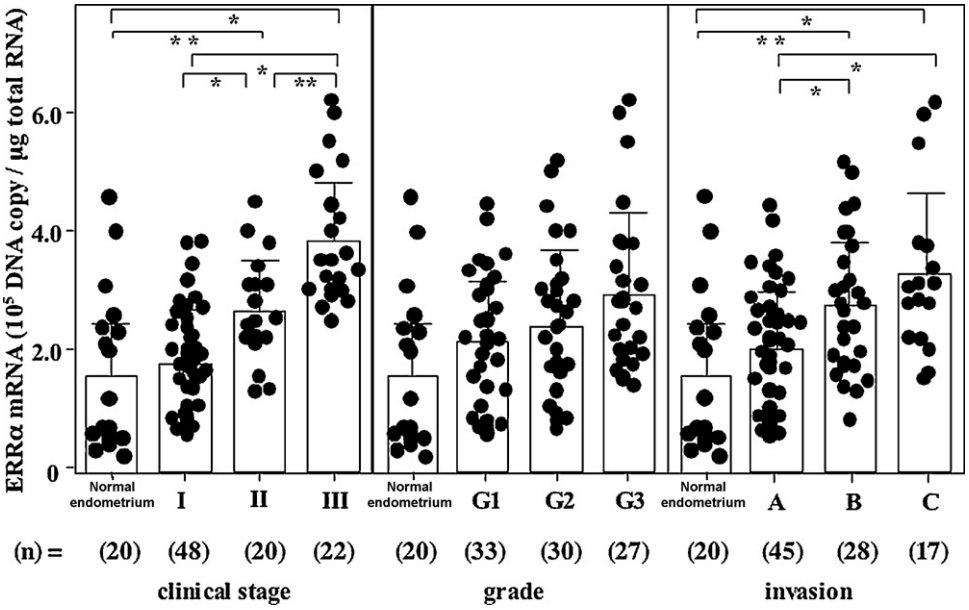

Fig. 3. Levels of ERRα mRNA in normal uterine endometria and uterine endometrial cancers classified according to clinical stages, histopathological types and myometrial invasion. Abbreviations of histopathological type and myometrial invasion were followed as in Fig. 1. Each level is the mean of 9 determinations. $^{*}p < 0.001$; $^{**}p < 0.05$.

3. Results

ERα and ERβ mRNA levels decreased with clinical stage, myometrial invasion and dedifferentiation, as shown in Figs. 1 and 2. ERRα mRNA levels increased with clinical stage and myometrial invasion, regardless of dedifferentiation, as shown in Fig. 3. On the other hand, ERRβ and ERRγ mRNA levels were extremely lower than ERRα mRNA levels, and ERRβ and ERRγ mRNA levels did not show any specific relation to clinical stage, myometrial invasion or dedifferentiation (data not shown).

All of the endometrial cancer specimens revealed strong staining for ERRα in the cancer cells. Positive staining for ERRα was in the nuclear of cancer cells. Immunohistochemical staining for ERRα of a representative case of moderately differentiated endometrioid adenocarcinoma of the uterine endometrium is shown in Fig. 4.

ERRα histoscores significantly correlated with ERRα mRNA levels as shown in Fig. 5. ERRα histoscores increased with clinical stage and myometrial invasion, regardless of dedifferentiation, as shown in Fig. 6.

4. Discussion

In the present study, ERα and ERβ expressions were down-regulated with tumor progression involving dedifferentiation and myometrial invasion. This may lead to the loss of estrogen dependency in growth by decreasing ERα and ERβ with tumor advancement in uterine endometrial cancers.

On the other hand, ERRα expression was up-regulated with tumor progression involving myometrial invasion, regardless of dedifferentiation. ERRs can bind to the steroid receptor coactivator family without any ligands, and drive transcription activity of the target genes [2]. The manner of ERRα and ERα gene expressions might show a competitive interaction associated with the

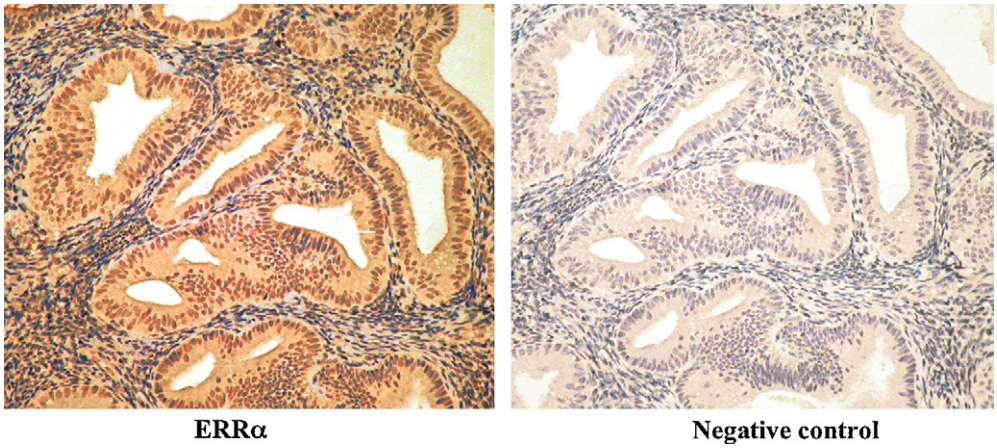

Fig. 4. Immunohistochemical staining for ERRα in uterine endometrial cancer (original magnification 200×). A representative case of moderately differentiated endometrioid adenocarcinoma of the uterine endometrium. Mouse anti-human ERRα antibody (Perseuse Proteomics) was used at dilutions of 1:125 as the primary antibody. Dark brown staining represents positive ERRα antigen.

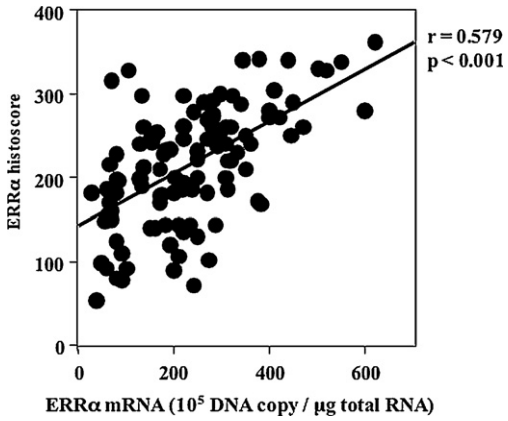

Fig. 5. Correlation of ERRα histoscores with ERRα mRNA levels in normal uterine endometria and uterine endometrial cancers. Correlation between ERRα histoscores in cancer cells with ERRα mRNA levels in uterine endometrial cancers. ERRα histoscores and ERRα mRNA levels were determined by immunohistochemistry and real-time RT-PCR, respectively. Each level is the mean of 9 determinations.

use of common cofactors [1] and loss of estrogen dependency. In this study, the competitive interaction of ERRα/ER expression associated with the use of common cofactors during loosing estrogen dependency might cause their expression manner. At last, the up-regulation of ERRα might be related to tumor growth and advancement, and a candidate for prognostic factors in uterine endometrial cancer. Target genes for ERRα including lactoferrin, medium-chain acyl coenzyme A dehydrogenase and osteopontin have been detected, but not those for tumor growth yet [3,4]. In breast cancer, ERRα immunoactivity was significantly associated with an increased risk of recurrence and adverse clinical outcome. It suggest that ERRα immunoactivity is a potent prognostic factor in human breast cancer [15]. ERRα expressed in uterine endometrial cancers suppresses estrogen responsive element-dependent transcriptional activity in the presence of estrogen. ERRα modulates estrogen-induced activity in estrogen-dependent uterine endometrial cancer [16]. It is speculated that ERRα is a candidate for prognostic factors in uterine endometrial cancer, although ERRs are not directly related to growth of uterine endometrial cancer.

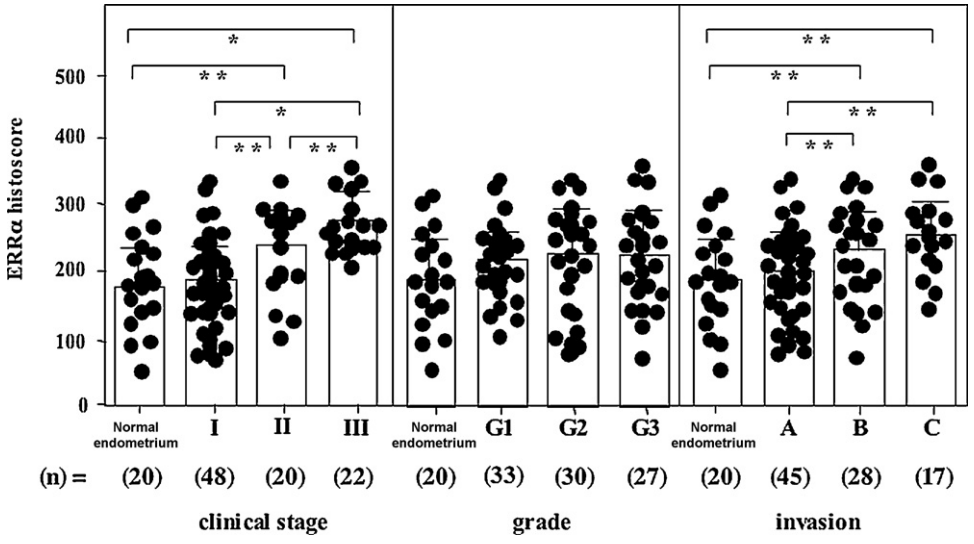

Fig. 6. ERRα histoscores in normal uterine endometria and uterine endometrial cancers classified according to clinical stages, histopathological types and myometrial invasion. The histoscores of ERRα were determined by immunohistochemistry. Abbreviations of histopathological type and myometrial invasion were followed as in Fig. 1. $^{*}p < 0.001$; $^{**}p < 0.05$.

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