研究テーマ

子宮内膜癌における ERα の特異的転写共役因子 p72 の発現の検討

— 子宮内膜癌における ERα 転写共役因子の発現検討 —

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乳癌、子宮内膜癌、卵巢癌の一部は、エストロゲン依存性に分化・増殖する。実際、乳癌、子宮内膜癌において、エストロゲンレセプター（ER）の発現は癌の治療法や予後を決定する上で重要な因子の一つになっている。エストロゲンの生理作用は、その特異的な核内レセプターERα、ERβを介した標的遺伝子の転写制御により発揮される。近年、核内レセプターによる転写開始には、RNAポリメラーゼIIを中核とした基本転写装置を仲介する転写共役因子群の存在が必須であることが示されてきた。最も解析されている転写共役因子であるSRC-1、TIF2、AIB1は、その相同性からファミリー（SRC-1 protein family）を形成している。

我々はこれまでに、AIB1に結合する因子のスクリーニングを行い、その結果72kDaのRNA結合蛋白p72を得た。p72は、ERα AF-1、SRC-1 protein familyと直接結合するが、他の核内レセプターとの結合は認められなかった。また、p72は、RNA転写共役因子であるSRAとも結合し、さらに、SRC-1 protein family、SRAと複合体を形成し、これらと協調してERα AF-1の転写活性を増強した。また、p72はERα AF-1アゴニストであるタモキシフェン存在下でもERαの転写活性を促進し、ERα AF-1特異的な転写共役因子として機能することが証明された。

子宮内膜癌では、病理組織学的に高分化なものでは、ER、PR（プロゲステロンレセプター）の発現頻度が高く、未分化なものではER、PRの発現頻度が低いこと、また、ER、PRの発現頻度が高いものほどホルモン療法が有用であることが知られている。しかしながら、子宮内膜癌において、ERとAIB1などの転写共役因子の発現との関係はまだ明らかでない。本研究は、子宮内膜癌におけるp72、AIB1、erbB-2、ERαの発現動態を検討し、ホルモン依存性腫瘍の分化・増殖のメカニズムをより詳細に解明することを目的とした。

子宮内膜癌症例において組織分化度の低下とともにERα、p72、AIB1の発現は低下し、対照的にerbB-2の発現は上昇していくことが定量的RT-PCRにより明らかになった。すなわち、ER、p72、AIB1の発現量が少ない低分化の組織では、代償的にerbB-2の発現が上昇しERα AF-1の転写活性を維持することが推測された。次に、リガンドとしてエストラジオールあるいはERα AF-1アゴニスト/AF-2アンタゴニストである4-ヒドロキサタモキシフェンの存在下で、p72、AIB1、erbB-2はERαの転写活性を協調的に促進することがルシフェラーゼ・アッセイにより明らかになった。これらの結果より、子宮内膜癌においてはp72、AIB1などの転写共役因子がerbB-2と協調的にERα AF-1活性としてERαの転写活性を促進すること、またこれがERα AF-1アゴニストのタモキシフェン使用による子宮内膜癌発症機序のひとつであることが示唆された。以上より、ERの量だけでなく、転写を調節する転写共役因子の量や種類がホルモン依存性腫瘍の増殖・分化・悪性度に関与し得ると考えられる。
Abstract

Objectives: To see how estrogens are involved in the growth of endometrial cancer with varying degrees of differentiation, we investigated the status of p72, a novel specific coactivator for estrogen receptor α (ERα) AF-1, AIB1, a steroid receptor coactivator amplified in breast cancer 1, erbB-2, a receptor tyrosine kinase, and ERα in endometrial cancer. Methods: Gene expression of ERα, p72, AIB1 and erbB-2 was measured in 26 samples of primary endometrial cancers by real-time RT-PCR and their in vivo cellular effects on the transactivation function of ERα were examined by a transient expression assay. Results: The mRNA levels of erbB-2 increased and those of ERα, p72 and AIB1 decreased with the loss of histological differentiations. Transient expression of p72, AIB1 and erbB-2 in human embryonic kidney 293T cells led to a synergistic promotion of the transactivation function of ERα in the presence of 17α-estradiol or 4-hydroxytamoxifen, an ERα AF-1 agonist/AF-2 antagonist, as a ligand. Conclusion: Estrogen action through ERα AF-1 might be exerted by the increased expression of the coactivators, p72 and AIB1, together with cross talk between erbB-2 and p72 to accelerate the transactivation of ERα AF-1 in endometrial cancer. These findings also suggest that the cooperative transactivation ERα AF-1 by the overexpression of p72, AIB1 and erbB-2 might be involved in tamoxifen-stimulated growth of endometrial cancer and that p72 could be considered as a prognostic marker in endometrial cancer.

Introduction

Estrogen binds to estrogen receptors (ERs) which belong to the nuclear receptor superfamily and function as a ligand-inducible transcriptional factor to control transcription of target genes [1-3]. The N-terminal A/B domain and the C-terminal E/F domain provide transactivation functions of ER. The autonomous activation function-1 (AF-1) in the A/B domain is constitutively active while AF-2 in the E/F domain is dependent on ligand binding [4]. A ligand bound ER forms a large complex to initiate transcription which is thought to contain basic transcriptional machinery and transcriptional coactivators [5]. CBP/p300 and SRC-1 family proteins (SRC-1/TIF2/AIB1) are known as cofactors to bind to ERα AF-2 in a ligand-dependent manner to promote transcription [6-9]. SRA is an RNA coactivator selective for ERα AF-1 [10]. Especially, AIB1 possesses a configuration that is to be phosphorylated by mitogen-activated protein kinase (MAPK) [11]. Recently, we have found that two DEAD-box proteins, p72 and p68, form a complex with SRC-1 family proteins and SRA, and function as specific coactivators for ERα AF-1 by directly binding to the ERα A/B domain [12-14]. The interaction of p72/68 with the ERα A/B domain was potentiated by phosphorylation of the Ser118 residue in the ERα A/B domain by MAPK, leading to the enhancement of ERα AF-1 activity [13-15].

Endometrial cancer is the most common female genital tract malignancy in the Western countries. The histologically more differentiated cases with high expression levels of sex steroid receptors, respond better to hormone treatment [16-18]. EGF is known to play a regulatory role in proliferation of endometrial cancer cells [19, 20]. Overexpression of erbB-2, a receptor tyrosine kinase that is similar to EGF receptor in structure, has been reported to be associated with poor survival in patients with endometrial cancer [21, 22]. ErbB-2 initiates its intracellular signal transduction by tyrosine-phosphorylating its intracellular domain and provides docking sites for signaling molecules [23]. The intracellular signaling induced by the phosphorylation of erbB-2 activates the MAPK cascade, which has been reported to be associated with cell proliferation, tumor progression and metastasis [23].

Recently, poorly differentiated breast cancer was shown to overexpress both AIB1 and erbB-2, which is accompanied by the lack of ER [11]. So far, however, the quantitative relationship among ERα, its transcriptional cofactors and erbB-2 in endometrial cancer has not been determined. In the present study, firstly, we investigated the expression levels of mRNA for ERα, p72, AIB1 and erbB-2 in endometrial cancers by real-time RT-PCR. Next, in order to test whether there is a cross talk between the cofactors, p72 and AIB1, and erbB-2 in the transactivation of ERα, we examined their in vivo cellular effects on the transactivation function of ERα by a transient expression assay.

Materials and Methods

Chemicals
An active metabolite of tamoxifen, 4-hydroxytamoxifen (OHT), and 17α-estradiol (E2) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of the highest grade commercially available.

Tissue samples
The endometrial cancer tissue specimens were obtained from 26 patients who underwent hysterectomy at the University of Tokyo Hospital. The mean age of the 26 patients was 55 years ranging from 36 to 79 years. Staging of tumors, based on the FIGO criteria [24], was as follows: 18 cases, stage I (T1, N0; tumor limited to corpus); 1 case, stage II (T2, N0; tumor involving cervix but not extending outside uterus); 5 cases, stage III (T3 or N1; tumor extending outside uterus, including spread to vagina, but remaining within the pelvis or metastases to regional lymph nodes); and 2 cases, stage IV (T4 or M1; tumor invading bladder or bowel mucosa or distant metastases). The histological subtype of all tumors was endometrioid adenocarcinoma [24]. The histological grading of differentiation of these endometrioid adenocarcinomas was as follows: 10 cases, well-differentiation (G1); 10 cases, moderately differentiation (G2); and 6 cases, poorly differentiation (G3) [24]. All of the patients provided informed consent for the research use of their samples. The biopsied tissue samples were snap-frozen in liquid nitrogen and stored at -70°C.
RT and real-time PCR
Total RNA was extracted from the frozen tissues using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). First-stand cDNA was synthesized in a reaction volume of 20 μl containing 1 μg total RNA by using ReverTra Dash (Toyobo, Tokyo, Japan) according to the manufacturer’s instructions. After the reverse transcription reaction, cDNA was amplified to determine p72, ERα, erbB-2 and AIB1 expression respectively by using the following PCR primer pairs: p72, 5′-GAC ACA AAT TTC GTA TCG TGG-3′ (sense), and 5′-GGC CTC TTC CAG CAC TTT GAT-3′ (antisense); ERα, 5′-AGC GTG TCT CCG AGC CCG CTG-3′ (sense), and 5′-GTT TTT ATC AAT GGT GCA CTG-3′ (antisense); erbB-2, 5′-TTG ACT CTG AAT GTC GGC CA-3′ (sense), and 5′-CCT TCG GAG GGT GCC AGT GG-3′ (antisense); and AIB1, 5′-ATA CTT GCT GGA TGG TGG ACT-3′ (sense), and 5′-TCC TTG CTC TTT TAT TTG ACG-3′ (antisense). Expression of these mRNA was normalized to RNA loading for each sample using GAPDH mRNA as an internal standard. The primers of GAPDH were used as follows: 5′-TGC GCT GTT GAA GTC AGA GGA GAC-3′ (sense) and 5′-GTC GCT GTT GAA GGA AGA GGA GAC-3′ (antisense).

Real-time PCR was performed using the LightCycler (Roche Applied Science, Mannheim, Germany) in 20 μl including 1.6 mM MgCl2, 2 μL LightCycler-FastStart Reaction Mix SYBR Green 1 (Roche Applied Science), 0.25 μM of each primer, and 50 ng cDNA from RT reactions as template. After an initial denaturation at 95 °C for 10 min, the amplification program for p72, ERα, erbB-2, AIB1 and GAPDH consisted of 35 cycles of denaturation for 15 sec at 95 °C, annealing for 8 sec at 64 °C, and extension for 11 sec at 72 °C. Finally, the temperature was raised gradually (0.2 °C/sec) from the annealing temperature to 95 °C for the melting curve analysis.

The samples were analyzed as follows. The concentrations of the samples were extrapolated from the standard curve by LightCycler software. Exogenous cDNA standards for p72, ERα, erbB-2, AIB1 and GAPDH were produced by inserting PCR products, which were generated using sample primers noted above and endometrial cancer tissue specimens cDNA as templates, into the pCR2.1 vector using the TOPO TA Cloning kit (Invitrogen Corp., Carlsbad, CA, USA). The concentration of each standard was determined by measuring the OD260, and the copy number was calculated. Relative expression levels of p72, ERα, erbB-2 and AIB1 were calculated by subtracting the signal threshold cycle (Ct) of the internal standard (GAPDH) from the Ct of p72, ERα, erbB-2 and AIB1.

Luciferase assay
Human embryonic kidney 293T cells were transfected using Lipofectin reagent (Invitrogen Corp.). A luciferase reporter plasmid containing CMV promoter (pRL-CMV) and estrogen response element with thymidine kinase promoter (pGL-ERE-tk) was co-transfected with the expression vectors indicated in the figure legend. A luciferase reporter assay was performed by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as described previously [13].

Statistical analysis
Data represent the mean ± SEM. The statistical analysis of relative mRNA expression levels of ERα, p72, AIB1, and erbB-2 was the mean ± Mann-Whitney U-test. P value less than 0.05 was considered statistically significant.

Results
Expression levels of mRNA of erbB-2, p72, AIB1 and ERα in endometrial cancers
Real-Time quantitative RT-PCR revealed that mRNA levels of erbB-2 (G1, 1.00 ± 0.09; G2, 2.67 ± 0.39; G3, 7.12 ± 1.32) were higher in poorly differentiated endometrial cancers compared with those in well-differentiated cases, whereas mRNA levels of ERα (G1, 1.00 ± 0.42; G2, 0.76 ± 0.25; G3, 0.24 ± 0.12), p72 (G1, 1.00 ± 0.20; G2, 0.70 ± 0.13; G3, 0.16 ± 0.08) and AIB1 (G1, 1.00 ± 0.32; G2, 0.80 ± 0.18; G3, 0.36 ± 0.09) decreased with the loss of histological differentiation (Fig. 1). No relationship was found between these mRNA levels and clinical stage.

Synergistic action of erbB-2 with p72 and AIB1 in the transactivation function of ERα
Luciferase assay revealed that the transient expression of erbB-2 promoted the transactivation function of ERα synergistically with p72 and AIB1 in the presence of E2 as a ligand (Fig. 2). The similar result was obtained with OHT, although the transcriptional activity of ERα with E2 was greater than that with OHT.

Discussion
In the present study, we demonstrated that mRNA levels of p72, a specific coactivator for ERα AF-1, AIB1 and ERα decreased with the loss of histological differentiation, whereas those of erbB-2 increased inversely. These findings led us to postulate that poorly differentiated endometrial cancer tissues seem to be in a condition where estrogen-independent ERα AF-1 activity is maintained by compensatory increase in the expression of erbB-2 despite smaller quantity of p72, AIB1 and ERα. It is also likely that potentiation of the transactivation of ERα AF-1 by erbB-2-activated MAPK phosphorylation of p72, AIB1 and ERα in itself may lead to estrogen-independent transactivation of target genes [11, 13-15].

Next, we showed that the transient expression of p72, AIB1 and erbB-2 synergistically promoted the transactivation function of ERα in the presence of E2 or OHT as a ligand. It is known that tamoxifen functions as an agonist to ERα AF-1 and an antagonist to AF-2 [13, 25]. In our study, the transcriptional activity of ERα with E2 was greater than that with OHT. The reason may be that E2-bound ERα is transactivated through both the AF-1 and AF-2, whereas OHT-bound ERα is transactivated through the AF-1 alone. Tamoxifen is thought to improve disease-free survival of the women with breast cancer, whereas it increases the risk of endometrial cancer, especially in estrogen-deficient postmenopausal women [26, 27]. In light of the paradoxical growth effects of
tamoxifen in endometrial tissues as opposed to the breasts, it is likely that ERα AF-1 activity varies in different tissues. By extension, it seems that ERα AF-1 activity might be enhanced as a result of an ERα AF-1 specific coactivator such as p72 being overexpressed in endometrial tissues where tamoxifen functions as an estrogen agonist. The existence of tissue specific cofactors could explain the difference of tissue specific ligand action. These results suggest that estrogen action through ERα AF-1 might be activated by the increased expression of p72 and its cross talk with erbB-2 in well differentiated endometrial cancer and that there might be compensatory increase in the expression of erbB-2 to maintain ERα AF-1 activity in poorly differentiated endometrial cancer. In addition, the cooperative transactivation of ERα AF-1 by the overexpression of p72, AIB1 and erbB-2 might explain the mechanisms underlying the growth of tamoxifen-induced endometrial cancer. Given the positive relationship between the degree of differentiation and the expression level of p72, this study further highlights p72 as a potential prognostic marker in various estrogen-related diseases.

References


![Fig.1](image1.png)

**Fig.1.** Real-time quantitative RT-PCR analysis of mRNA levels of ERα, p72, AIB1 and erbB-2 in endometrial cancers. The mRNA levels were compared among the differentiation grades (G1, G2 and G3). The values represent the mean ± SEM of relative ratios of the expression levels.

*, P < 0.05, **, P < 0.01 vs. G1 by Mann-Whitney U-test.

![Fig.2](image2.png)

**Fig.2.** Cooperative promotion of transactivation of ERα by the overexpression of p72, AIB1 and erbB-2. 293T cells were transfected with ERα (HEGO) (0.1μg), pGL-ERE-tk (0.5μg), pRL-CMV(10ng), pcDNA-p72 (0.3μg), pcDNA-AIB1 (0.3μg) and erbB-2 plasmid under the SV40 promoter (0.3μg) in the presence of E2 or OHT at 10^{-8}M, and the cell extracts were used for luciferase assay. Results are shown as the mean ± SD. In the presence of E2 or OHT, p72, AIB1 and erbB-2 caused an ultimate potentiation of the ERα transactivation function (Lanes 16 and 24).

＜刊行（投稿）に関する一覧表＞
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