SPANDIDOS PUBLICATIONS

Analysis of the status of the novel estrogen receptor α (ERα) coactivator p72 in endometrial cancer and its cross talk with erbB-2 in the transactivation of ERα

LIN ZHAO[1*], MICHIKO WATANABE[1*], TETSU YANO[1], JUNN YANAGISAWA[2], SHUNSUKE NAKAGAWA[1], HAJIME OISHI[1], OSAMU WADA-HIRAIKE[1], KATSUTOSHI ODA[1], TAKEO MINAGUCHI[1], TOSHIHARU YASUGI[1], SHIGEAKI KATO[3] and YUJI TAKETANI[1]

[1]Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo 113-8655; [2]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba Science City, Ibaraki 305-8572; [3]Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113-0032 Japan

Received December 27, 2007; Accepted February 19, 2008

Abstract. To determine 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) activation function-1 (AF-1), AIB1, a steroid receptor coactivator amplified in breast cancer 1, erbB-2, a receptor tyrosine kinase, and ERα in endometrial cancer. 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. The mRNA levels of erbB-2 increased and those of ERα, p72 and AIB1 decreased with the loss of histological differentiation. 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. In 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 of ERα AF-1 by the overexpression of p72, AIB1 and erbB-2 might be involved in tamoxifen-stimulated growth of endometrial cancer.

Correspondence to: Dr Tetsu Yano, Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
E-mail: tetu-tky@umin.ac.jp

*Contributed equally

Key words: estrogen, receptor, p72, cofactor, transactivation

Introduction

Estrogen binds to estrogen receptors (ERs) which belong to the nuclear receptor superfamily and function as ligand-inducible transcriptional factors 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, thought to contain basic transcriptional machinery and transcriptional cofactors, to initiate transcription (5). CBP/p300 and SRC-1 family proteins (SRC-1/TIF2/AIB1) are known as cofactors which 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). In particular, AIB1 possesses a configuration that is phosphorylated by mitogen-activated protein kinase (MAPK) (11). Recently, we found that two DEAD-box proteins, p72 and p68, form a complex with SRC-1 family proteins and SRA, functioning 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 Western countries. Histologically more differentiated cases with high expression levels of sex steroid receptors respond best to hormone treatment (16-18). EGF is known to play a regulatory role in the proliferation of endometrial cancer cells (19,20). Overexpression of erbB-2, a receptor tyrosine kinase similar to the 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. This was accompanied by the lack of ERs (11). So far, however, the quantitative relationship between ERα, its transcriptional cofactors and erbB-2 in endometrial cancer has not been determined.

In the present study, we first investigated the mRNA expression levels of ERα, p72, AIB1 and erbB-2 in endometrial cancers by real-time RT-PCR. Next, in order to test whether there is cross talk between the p72 and AIB1 cofactors 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-hydroxy-tamoxifen (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. Endometrial cancer tissue specimens were obtained from 26 patients who underwent hysterectomy at the University of Tokyo Hospital. The study was approved by the Institutional Review Board for the University of Tokyo. All patients gave written informed consent for the research use of their samples. The mean age of the 26 patients was 55 years (range 36-79). Staging of tumors, based on the FIGO criteria (24), was as follows: 18 cases in stage I (T_1, N_0; tumor limited to corpus); 1 case in stage II (T_2, N_0; tumor involving cervix but not extending outside uterus); 5 cases in stage III (T_3 or N_1; tumor extending outside uterus and including dissemination to vagina, but remaining within pelvis or metastases to regional lymph nodes); and 2 cases in stage IV (T_4 or M_1; tumor invading bladder or bowel mucosa or distant metastases). The histological subtype of all tumors was endometrioid adenocarcinoma (24). The histological grading of the differentiation of these endometrioid adenocarcinomas was as follows: 10 cases, well-differentiated (G1); 10 cases, moderately-differentiated (G2); 6 cases, poorly-differentiated (G3) (24). 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 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 using the following PCR primer pairs: p72, 5'-GAC CAC AAG TTG ATC CAA CTA-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); 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 mRNAs was normalized to RNA loading for each sample using GAPDH mRNA as an internal standard. The primers of GAPDH were as follows: 5'-TCC ATG ACA ACT TTG GTA TCG TGG-3' (sense) and 5'-GTC GCT GTT GAA GTC AGA GGA GAC-3' (antisense).

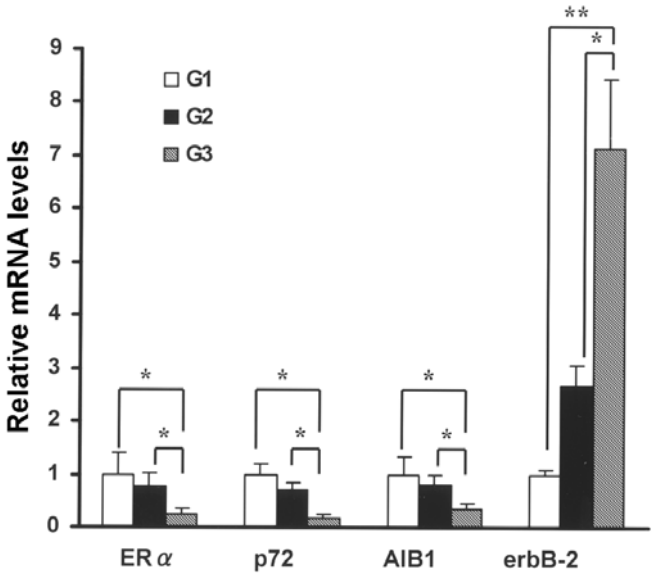

Figure 1. Real-time quantitative RT-PCR analysis of ERα, p72, AIB1 and erbB-2 mRNA levels in endometrial cancers. mRNA levels of the differentiation grades (G1, G2 and G3) were compared. Values represent the mean ± SEM of the relative ratios of the expression levels. $^{*}P<0.05$, $^{**}P<0.01$ vs. G1 (Mann-Whitney U test).

Real-time PCR was performed with the LightCycler (Roche Applied Science, Mannheim, Germany) in 20 μl consisting of 1.6 mM $MgCl_2$, 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 a 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 according to the following procedure. 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, generated using the sample primers noted above, and endometrial cancer tissue specimen cDNA templates into the pCR2.1 vector with 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 (C_T) of the internal standard (GAPDH) from the C_T 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

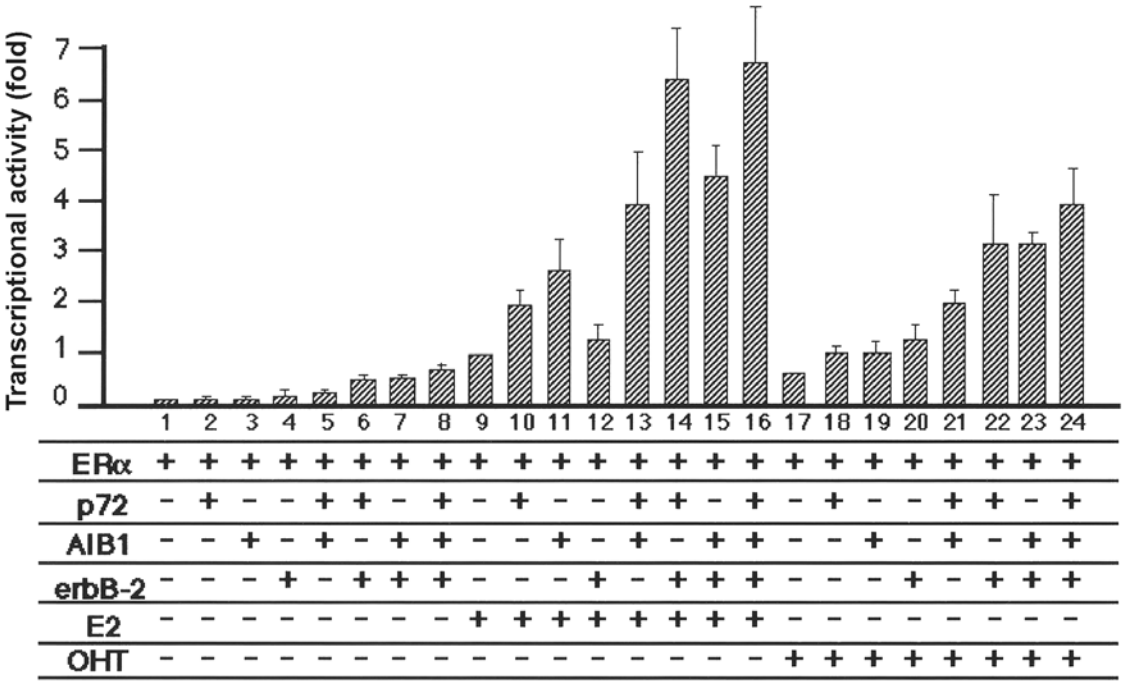

Figure 2. Cooperative promotion of ERα transactivation by the overexpression of p72, AIB1 and erbB-2. 293T cells were transfected with ERα (HEGO) (0.1 μg), pGL-ERE-tk (0.5 μg), pRL-CMV (10 ng), pcDNA-p72 (0.3 μg), pcDNA-AIB1 (0.3 μg) and erbB-2 plasmid under the SV40 promoter (0.3 μg) in the presence 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).

kinase promoter (pGL-ERE-tk) was co-transfected with the expression vectors indicated in the legend of Fig. 2. A luciferase reporter assay was performed 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 performed by the Mann-Whitney U test. A P-value <0.05 was considered statistically significant.

Results

mRNA expression levels 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 than 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). A similar result was obtained with OHT, although the transcriptional activity of ERα with E2 was greater than it was with OHT.

Discussion

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

We additionally 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 as an antagonist to AF-2 (13,25). In our study, the transcriptional activity of ERα with E2 was greater than it was with OHT. The reason may be that E2-bound ERα is transactivated through both AF-1 and AF-2, whereas OHT-bound ERα is transactivated through AF-1 alone. Tamoxifen is thought to improve disease-free survival in women with breast cancer, whereas it increases the risk of endometrial cancer, especially in estrogen-deficient post-menopausal 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. Thus, 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 in 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 in poorly-differentiated endometrial cancer there might be a compensatory increase in the expression of erbB-2 to maintain ERα AF-1 activity. In addition, the co-operative 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 degree of differentiation and the expression level of p72, this study further highlights p72 as a potential prognostic marker in various estrogen-related diseases.

Acknowledgements

We thank Drs T. Yamauchi and T. Kadowaki for supplying the erbB-2 expression vectors, and Dr P. Chambon for supplying ERα (HEGO). This work was supported in part by a Grant-in-Aid for priority areas from the Ministry of Education, Science, Sports and Culture of Japan.

References

1. Beato M, Herrlich P and Schutz G: Steroid hormone receptors: many actors in search of plot. Cell 83: 851-857, 1995.
2. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P and Evans RM: The nuclear receptor superfamily: the second decade. Cell 83: 835-839, 1995.
3. Chambon P: A decade of molecular biology of retinoic acid receptors. FASEB J 10: 940-954, 1996.
4. Lees JA, Fawell SE and Parker MG: Identification of two transactivation domains in the mouse oestrogen receptor. Nucleic Acids Res 17: 5477-5488, 1989.
5. Freedman PL: Increasing the complexity of coactivation in nuclear receptor signaling. Cell 97: 5-8, 1999.
6. Onate SA, Tsai SY, Tsai MJ and O'Malley BW: Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270: 1354-1357, 1995.
7. Voegel JJ, Heine MJ, Zechel C, Chambon P and Gronemeyer H: TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J 15: 3667-3675, 1996.
8. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM and Meltzer PS: AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277: 965-968, 1997.
9. Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK and Rosenfeld MG: The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature 387: 677-684, 1997.
10. Lanz RB, McKenna NJ, Onate SA, Albrecht U, Wong J, Tsai SY, Tsai MJ and O'Malley BW: A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. Cell 97: 17-27, 1999.
11. Bouras T, Southey MC and Venter DJ: Overexpression of the steroid receptor coactivator AIB1 in breast cancer correlates with the absence of estrogen and progesterone receptors and positivity for p53 and HER2/*neu*. Cancer Res 61: 903-907, 2001.
12. Lamm GM, Nicol SM, Fuller PF and Lamond AI: p72: a human nuclear DEAD box protein highly related to p68. Nucleic Acids Res 24: 3739-3747, 1996.
13. Watanabe M, Yanagisawa J, Kitagawa H, Takeyama K, Ogawa S, Arao Y, Suzawa M, Kobayashi Y, Yano T, Yoshikawa H, Masuhiro Y and Kato S: A subfamily of RNA binding DEAD-box proteins acts as an estrogen receptor alpha coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator, SRA. EMBO J 20: 1341-1352, 2001.
14. Endoh H, Maruyama K, Masuhiro Y, Kobayashi Y, Goto M, Tai H, Yanagisawa J, Metzger D, Hashimoto S and Kato S: Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha. Mol Cell Biol 19: 5363-5372, 1999.
15. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D and Chambon P: Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270: 1491-1494, 1995.
16. Podratz KC, O'Brien PC, Malkasian GD Jr, Decker DG, Jefferies JA and Edmonson JH: Effects of progestational agents in treatment of endometrial carcinoma. Obstet Gynecol 66: 106-110, 1985.
17. Creasman WT, Soper JT, McCarty KS Jr, McCarty KS Sr, Hinshaw W and Clarke-Pearson DL: Influence of cytoplasmic steroid receptor content on prognosis of early stage endometrial carcinoma. Am J Obstet Gynecol 151: 922-932, 1985.
18. Kauppila AJ, Isotalo HE, Kivinen ST and Vihko RK: Prediction of clinical outcome with estrogen and progestin receptor concentrations and their relationships to clinical and histopathological variables in endometrial cancer. Cancer Res 46: 5380-5384, 1986.
19. Pearl ML, Talavera F, Gretz HF III, Roberts JA and Menon KM: Mitogenic activity of growth factors in the human endometrial adenocarcinoma cell lines HEC-1-A and KLE. Gynecol Oncol 49: 325-332, 1993.
20. Lelle RJ, Talavera F, Gretz H, Roberts JA and Menon KM: Epidermal growth factor receptor expression in three different human endometrial cancer cell lines. Cancer 72: 519-525, 1993.
21. Hetzel DJ, Wilson TO, Keeney GL, Roche PC, Cha SS and Podratz KC: HER-2/neu expression: a major prognostic factor in endometrial cancer. Gynecol Oncol 47: 179-185, 1992.
22. Kohlberger P, Loesch A, Koelbl H, Breitenecker G, Kainz C and Gitsch G: Prognostic value of immunohistochemically detected HER-2/neu oncoprotein in endometrial cancer. Cancer Lett 98: 151-155, 1996.
23. Yamauchi T, Yamauchi N, Ueki K, Sugiyama T, Waki H, Miki H, Tobe K, Matsuda S, Tsushima T, Yamamoto T, Fujita T, Taketani Y, Fukayama M, Kimura S, Yazaki Y, Nagai R and Kadowaki T: Constitutive tyrosine phosphorylation of ErbB-2 via Jak2 by autocrine secretion of prolactin in human breast cancer. J Biol Chem 275: 33937-33944, 1999.
24. Pecorelli S, Benedet JL, Creasman WT and Shepherd JH: FIGO staging of gynecologic cancer. 1994-1997 FIGO Committee on Gynecologic Oncology. International Federation of Gynecology and Obstetrics. Int J Gynaecol Obstet 65: 243-249, 1999.
25. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA and Greene GL: The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95: 927-937, 1998.
26. Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Silfversward C, Skoog L, Somell A, Theve T, Wilking N, Askergren J and Hjalmar ML: Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet 1: 117-120, 1989.
27. Fisher B, Costantino JP, Redmond K, Fishe ER, Wickerham DL and Cronin WM: Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst 86: 527-537, 1994.