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Article

Identification of Genes Differentially Expressed in the MCF-7 Cells Treated with Mitogenic Estrogens

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Estrogens, a group of steroid compounds functioning as the primary female sex hormone, play an important role in the development and progression of breast cancer. In this study, using a novel annealing control primer-based GeneFishing PCR technology, five differentially expressed genes (DEGs), expressed using 10 nM mitogenic estrogens, 17β-estradiol (E2) and 16α-hydroxyestrone (16α-OHE1), were selected from the estrogen receptor (ER)-positive MCF-7 human breast cancer cells. The DEGs, *MRPL42, TUBA1B, SSBP1, KNCT2*, and *RUVBL1*, were identified by comparison with the known genes via direct sequencing and sequence homology search in BLAST. Quantitative real-time PCR data showed that two DEGs, tubulin α1b and kinetochore associated 2, were greater than 2-fold upregulated by E2 or 16α-OHE1. Both genes could be new biomarkers for the treatment and prognosis of cancers, and further study may provide insights into the molecular mechanisms underlying development and progression of breast cancer.

Key words : *differentially expressed genes, 17β-estradiol, estrogen receptor-positive MCF-7 human breast cancer cells, 16α-hydroxyestrone, kinetochore associated 2, tubulin α1b*

Estrogens are a group of steroid compounds functioning as the primary female sex hormone. Menopause is the physiological cessation of the menstrual cycle associated with aging in women, resulting in the decrease of the estrogen production. Estrogen deficiency leads to the post-menopausal symptoms such as flashes, insomnia, depression and osteoporosis. Post-menopausal women are at an increased risk of osteoporosis, and many studies have reported that one reason for this risk is the dramatic change in the level of female sex hormones, especially estrogens. HRT can increase the bone mineral density and, as a result, decrease the skeletal fragility [Delmas, 1997]. Unfortunately, HRT increases cardiovascular events in the postmenopausal women [Grady *et al*., 2002] and a long-term compliance to HRT is limited by side effects such as breast cancer, the risk of which increases after prolonged treatments.

Estrogens play an important role in the development and progression of breast cancer [McGuire *et al*., 1976; Clemons and Goss, 2001]. Despite the considerable efforts to identify the genes involved in the molecular mechanisms underlying the estrogen-mediated breast cancer, conventional differential display-methods are labor-intensive and lead to a high degree of false positives. A novel ACP-based differential display-RT-PCR technology regulated by an ACP has been used to identify the DEGs [Ryu *et al*., 2007; Hwang *et al*., 2004]. The basis of the ACP technology is the unique tripartite structure of a specific oligonucleotide primer, which contains distinct 3'-end and 5'-end regions separated by a regulator, as well as the interactions of each portion of this primer during the two-stage PCR [Kim *et al*., 2004]. The ACP-based PCR system facilitates the identification of DEGs from the samples displaying low mRNA levels without generating false positives [Hwang *et al*., 2004].

Recently, Kim *et al*. [2005] reported that two mitogenic estrogens, 17β-estradiol (E2) and 16α-hydroxyestrone (16α-OHE1), induced the proliferation of the estrogen

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Abbreviations: ACP, annealing control primer; DC, dextran/charcoal; DEG, differentially expressed gene; DMEM, Dulbecco's Modified Eagle's Medium; E2, 17β-estradiol; ER, estrogen receptor; FBS, fetal bovine serum; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HDAC, histone deacetylase; HRT, hormone replacement therapy; 2-ME, 2-methoxyestradiol; 16α-OHE1, 16α-hydroxyestrone; PR, progesterone receptor.

receptor (ER)-positive MCF-7 human breast cancer cells at 10 nM with greater than 20-fold induction of the PR transcript, which is a classical example for an ER-mediated gene. This suggests that the mitogenic mechanisms of E2 and 16α-OHE1 could stem from a direct genomic action via the activation of ER. Therefore, in the present study, to identify the genes selectively expressed in MCF-7 cells by the mitogenic estrogens, E2 and 16α-OHE1, the gene expression patterns were examined using the ACP-based GeneFishing PCR technology.

Materials and Methods

Cell culture. Human MCF-7 breast cancer cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Hyclone Laboratories, South Logan, UT), 100 U/mL of penicillin, and 100 mg/mL streptomycin with a change of the medium every 3 days.

Estrogens treatment. The MCF-7 cells were plated into 96-well plates at a density of 4,000 cells per well in DMEM with 10% FBS and incubated for 24 h. The medium was replaced with a phenol red-free DMEM containing 5% DC-treated FBS to remove the endogenous steroids in the serum and exclude the weak estrogen-agonist activity of the phenol red [Darbre *et al.*, 1983; Ernst *et al.*, 1989]. After 24 h, the cells were incubated with 10 nM of each of the E2 metabolites (Steraloids Inc., Wilton, NH).

Isolation of total RNA. Using the TRIzol reagent (Life technologies), total RNA was isolated from the MCF-7 cells treated with each E2 metabolite for 24 h. The homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of the nucleoprotein complexes. After the addition of 0.2 volume of chloroform, the samples were shaken vigorously for 15 s, incubated for 3 min, and centrifuged at 12,000×g for 15 min at 4°C. The total RNA remaining in the upper aqueous phase was precipitated by mixing with an equal volume of isopropanol. The mixtures were incubated for 10 min and centrifuged at 12,000×g for 10 min at 4°C. The total RNA pellet was washed with 75% ethanol, dried, and dissolved in the RNase-free water. The concentration and purity of total RNA were calculated based on the difference in the absorbance at 260 and 280 nm.

First-strand cDNA Synthesis. Reverse transcriptase was used to synthesize the first-strand cDNAs from total RNAs . Reverse transcription was performed for 1.5 h at 42°C in a final reaction volume of 20 μL containing 3 μg of the purified total RNA, 4 μL of 5×reaction buffer (Promega, Madison, WI), 5 μL of dNTPs (each 2 mM), 2 μL of 10 μM dT-ACP1 (5'-CGTGAATGCTGCGACTA CGATIIIIIT(18)-3'), 0.5 μL of RNasin RNase Inhibitor (40 U/μL; Promega), and 1 μL of Moloney murine leukemia virus reverse transcriptase (200 U/μL; Promega). First-strand cDNAs were diluted by the addition of 80 μL of the ultra-purified water for the GeneFishingTM PCR, and stored at −20°C until use.

ACP-based GeneFishing™ PCR. DEGs were screened by the ACP-based PCR method [Kim *et al.*, 2004] using the GeneFishing™ DEG kits (Seegene, Seoul, Korea). Briefly, the second-strand cDNA synthesis was conducted at 50°C during one cycle of the first-stage PCR in a final reaction volume of 20 μL containing 3-5 μL (about 50 ng) of the diluted first-strand cDNA, 1 μL of dT-ACP2 (10 μM), 1 μL of 10 μM arbitrary ACP, and 10 μL of 2 ×Master Mix (Seegene). The PCR protocol for the second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After completion of the second-strand DNA synthesis, the second-stage PCR amplification protocol was carried out as follows; 40 cycles at 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, and a 5-min final extension at 72°C. The amplified PCR products were separated in the 2% agarose gel stained with ethidium bromide.

Direct Sequencing. The differentially expressed bands were re-amplified and extracted from the gel using the GENCLEAN II Kit (Q-BIO gene, Carlsbad, CA), and directly sequenced with the ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA). Complete sequences were determined by searching for similarities using a BLAST program (http://www.ncbi.nlm.nih.gov).

Primer design and real-time PCR. To set the most suitable PCR amplification conditions, the sequences of the primers were determined by an on-line primer design program [Rozen and Skaletsky, 2000]. The primer sets used in this study are shown in Table 1. First-strand cDNA was synthesized with 1 μg of total RNAs and 1 μM of oligo-dT_{15} primer using the Omniscript Reverse Transcriptase (Qiagen, Valencia, CA). SYBR Green-based quantitative PCR amplification was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, La Jolla, CA) with the 1 : 50 diluted first-strand cDNA and 20 pmole of the primers according to the manufacturer's protocols. The PCR reaction consisted of initial denaturation at 94°C for 3 min, 3-step cycling (40 cycles) at 94°C for 40 s, 60°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. All reactions were run in triplicates, and the data were analyzed using the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001]. To assess the

Table 1. Primer sequences used in this study

Gene	Forward	Reverse
MRPL42	5'-atg cta cca ccc ttc tgt gg-3'	5'-tgt gat acc gtc cat gag ga-3'
TUBA1B	5'-gcc cta caa ctc cat cct ca-3'	5'-gtc aac att cag ggc tcc at-3'
SSBP1	5'-aag atc cct gaa tcg tgt gc-3'	5'-gtt gct tgt cgc ctc aca t-3'
KNTC2	5'-agt tta atc ccg agg ctg gt-3'	5'-cag gtg ctt gtg ttt ctc ca-3'
RUVBL1	5'-gcc agc taa tga agc caa ag-3'	5'-cct cag tgc ctc tga tga ca-3'
GAPDH	5'-GTC AGT GGT GGA CCT GAC CT-3'	5'-agg ggt cta cat ggc aac tg-3'

differential transcript expression among the groups, GAPDH was used as the control gene. Significance was determined by Student's *t*-test of Microsoft Excel with a GAPDH-normalized $2^{-\Delta\Delta CT}$ value, and the expression differences were considered significant when $P<0.05$.

Results

Identification of up-regulated genes by the treatment of E2 or 16α-OHE1 in MCF-7 cells. To identify the estrogen-dependently expressed genes, the MCF-7 cells were cultured in a phenol red-free DMEM with 5% DC-treated FBS and treated with 10 nM of E2 or 16α-OHE1 under the serum-free condition. After the incubation for 24 h, the DEGs were observed using the ACP-based GeneFishing PCR technology. From 20 GPs, 5 DEGs that were up-regulated by E2 or 16α-OHE1 were identified (Fig. 1). Between GP 16 and GP 20, no DEGs were found (data not shown).

Sequence homology search for DEGs. Five differentially expressed transcripts, *MRPL42, TUBA1B, SSBP1, KNCT2*, and *RUVBL1*, were identified by comparison with the known genes via direct sequencing and sequence homology search in BLAST. The sequence similarities and characterizations of these five DEGs are summarized in Table 2.

Confirmation of DEGs expression levels by quantitative real-time PCR. To confirm the efficacy of the ACP system and further determine the estrogen-dependent expression patterns of the DEGs identified, SYBR Green-based quantitative real-time PCR was performed as described in Materials and Methods. Under the serum-free condition, *TUBA1B, SSBP1*, and *KNTC2* were significantly up-regulated by E2 or 16α-OHE1, consistent with the results of ACP-based PCR; however, *MRPL42* and *RUVBL1* were significantly up-regulated only in the cells treated with 16α-OHE1 (Fig. 2). Interestingly, the upregulation of the *TUBA1B* and *KNTC2* by E2 or 16α-OHE1 were greater than 2-fold.

Discussion

E2 is the most biologically active estrogen in the breast tissue and its administration in the rodent models has shown to be carcinogenic. Interestingly, its hydroxylated metabolites showing the estrogenic activities are also carcinogenic. The estrogenic activity of 16α-OHE1 has been proposed to be more potent than that of E2 [Fishman *et al.*, 1980]. In a population of women with breast cancer, abnormal 16α-hydroxylation of E2 has been observed [Schneider *et al.*, 1982], and the activity of 16α-hydroxylase, the enzyme involved in the formation of 16α-OHE1, increased [Fishman *et al.*, 1984]. Since 16α-OHE1 with the potential to induce the proliferation of the MCF-7 human breast cancer cells has been shown to covalently bind to ER, the covalent modification of ER by 16α-OHE1 was suggested be one mechanism of the malignant transformation in the estrogen target tissues [Swaneck and Fishman, 1988; Gupta *et al.*, 1998; Lewis *et al.*, 2001].

Recently, Kim *et al.* [2005] showed that E2 and 16α-OHE1 have highly mitogenic estrogen activities at a low concentration (10 nM), suggesting these mitogenic mechanisms of E2 and 16α-OHE1 could stem from their direct genomic action via the activation of ER. Therefore, in the present study, the genes selectively expressed by E2 or 16α-OHE1 in the ER-positive MCF-7 cells were identified using a novel ACP-based GeneFishing PCR technology. Five DEGs, *MRPL42, TUBA1B, SSBP1, KNCT2*, and *RUVBL1*, were identified by comparison with the known genes, and their transcript expression patterns were confirmed by SYBR Green-based quantitative real-time PCR. Under the serum-free condition, *TUBA1B* and *KNTC2* were up-regulated greater than 2-fold by 10 nM of E2 or 16α-OHE1 in the MCF-7 cells *per se*.

Tubulin, the structural protein of microtubules, is a heterodimer composed of two 50-kD acidic monomers, α- and β-tubulin, that polymerize to form microtubules, a component of the cytoskeletal system. A recent study

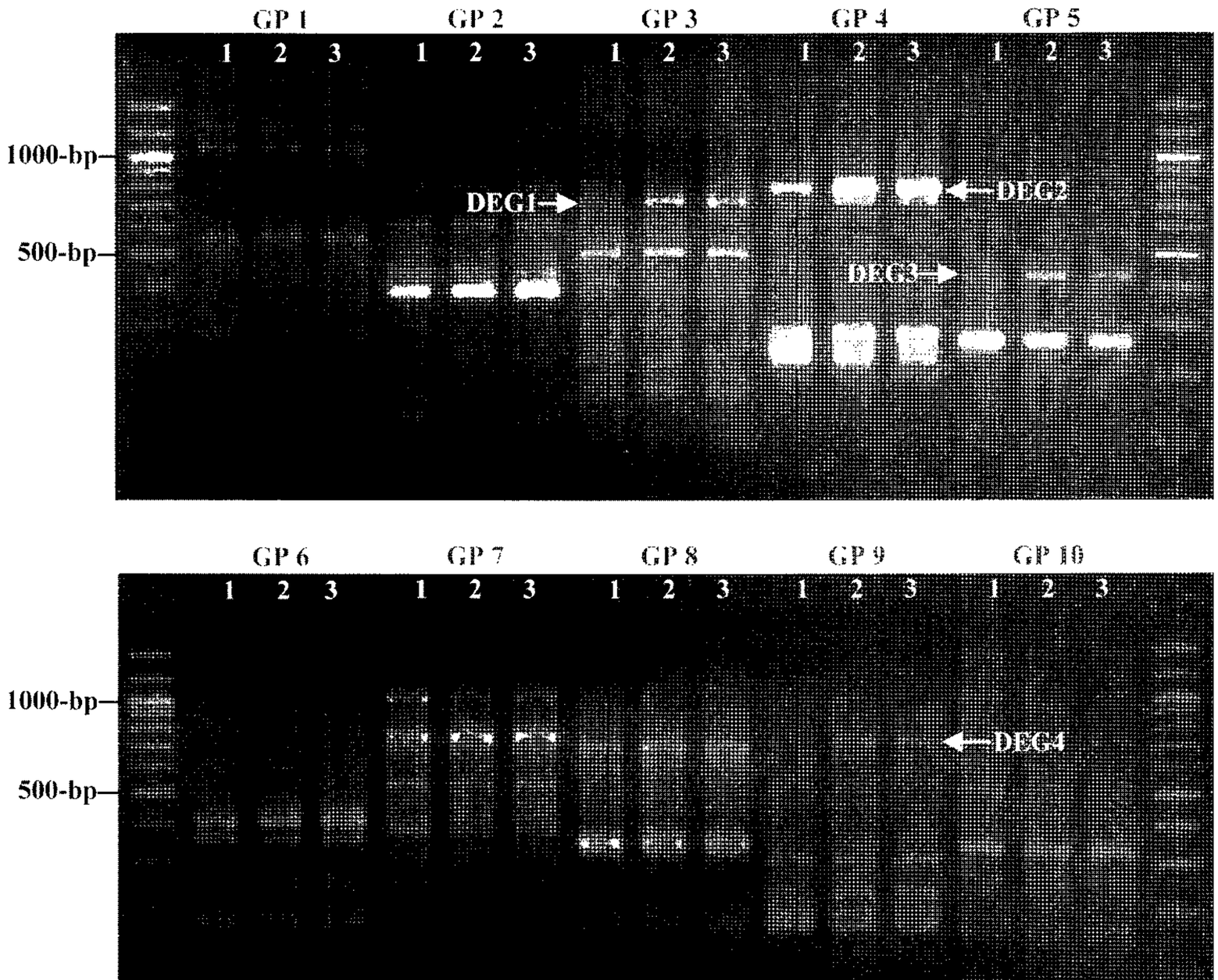

Fig. 1. Identification of mitogenic estrogen-regulated DEGs in MCF-7 cells. The mRNA expression profiling in MCF-7 cells (lane 1) was compared with that of cells treated with either 10 nM of E2 (lane 2) or 16α-OHE1 (lane 3). Between GP 1 and 15, 5 DEGs (DEG1 to 5) were identified to be up-regulated by both estrogens.

showed that the expression level of HDAC 6 mRNA/protein and the cell motility increased in the MCF-7 cells treated with E2 [Saji *et al.*, 2005]. In addition, the accumulation of HDAC also caused the deacetylation of α-tubulin. Together with the up-regulation of *TUBA1B* by the mitogenic estrogens, this result suggests that E2 can regulate the expressions of HDAC6 and tubulin isotype(s), and consequently affect the survival of the breast cancer cells. Moreover, the treatment of the prostate cancer cells with diethylstilbestrol and 2-ME suppressed the levels of the transcripts and the protein for β-tubulin isotype IVa [Montgomery *et al.* 2005]. In addition, upon the 2-ME treatment, the levels of acetylated and detyrosinated tubulins decreased in the wild type of the human breast cancer cells, while remaining unchanged in the 2-ME-resistant cells [Goekmen-Polar *et al.* 2005]. Interestingly, anti-cancer drug-resistant MCF-7 cells contain significantly higher amounts of tyrosinated α-tubulin than do the wild-type cells, suggesting that the level of certain isotype of tubulin becomes elevated in the drug-resistant cells [Banerjee, 2002]. In other words, the regulations of the tubulin gene expression and the microtubule dynamics by targeting (or binding) the tubulins could be promising ways to treat cancers [Sengupta and Thomas, 2006].

Table 2. Sequence homology search for DEGs

DEG No.	Sequence homology search
DEG1	gi|26667170|ref|NM_172177.1| **Homo sapiens mitochondrial ribosomal protein L42 (MRPL42)**, nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA Length=2073 Score=1231 bits (621), Expect=0.0 Identities=627/629 (99%), Gaps=0/629 (0%) Strand=Plus/Plus
DEG2	gi|57013275|ref|NM_006082.2| **Homo sapiens tubulin, alpha 1b (TUBA1B)**, mRNA Length=1771 Score=1360 bits (686), Expect=0.0 Identities=688/689 (99%), Gaps=0/689 (0%) Strand=Plus/Plus
DEG3	gi|4507230|ref|NM_003143.1| **Homo sapiens single-stranded DNA binding protein 1 (SSBP1)**, mRNA Length=628 Score=644 bits (325), Expect=0.0 Identities=334/336 (99%), Gaps=1/336 (0%) Strand=Plus/Plus
DEG4	gi|23274184|gb|BC035617.1| **Homo sapiens kinetochore associated 2 (KNTC2)**, mRNA (cDNA clone MGC:45222 IMAGE:5164424), complete cds Length=2140 Score=1191 bits (601), Expect=0.0 Identities=622/622 (100%), Gaps=0/622 (0%) Strand=Plus/Plus
DEG5	gi|4506752|ref|NM_003707.1| **Homo sapiens RuvB-like 1 (E. coli) (RUVBL1)**, mRNA Length=1750 Score=1281 bits (646), Expect=0.0 Identities=646/646 (100%), Gaps=0/646 (0%) Strand=Plus/Plus

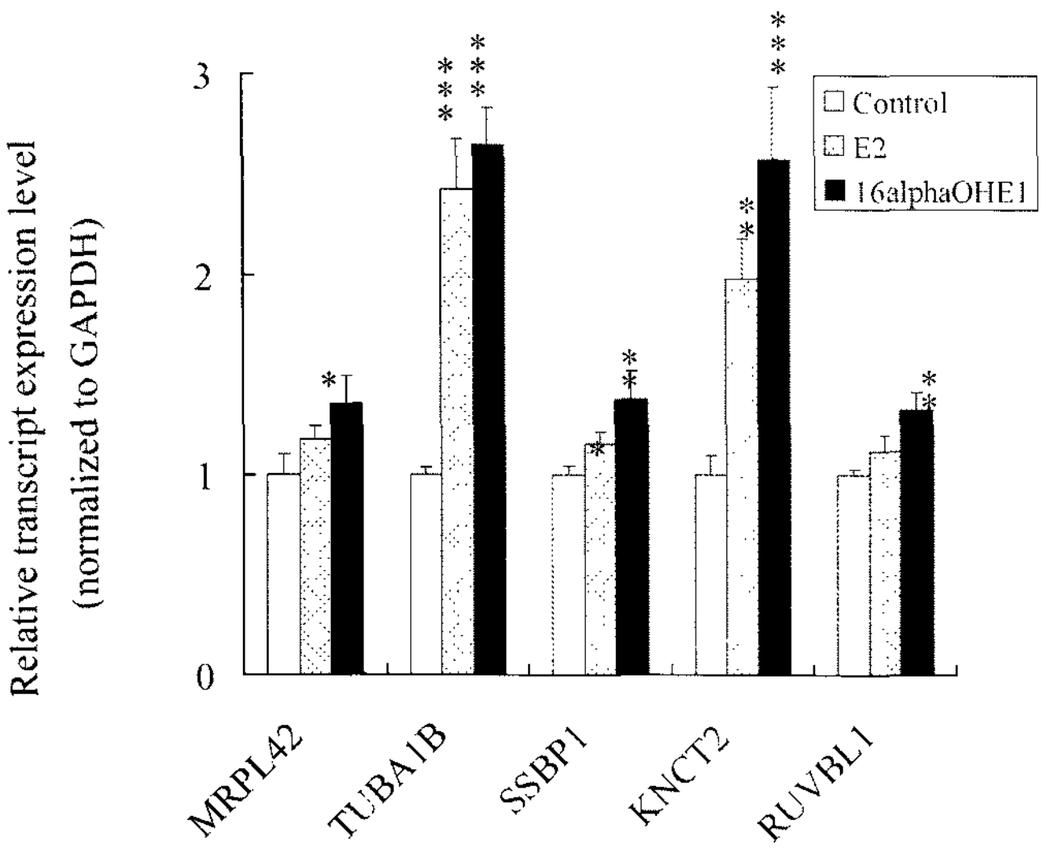

Fig. 2. Evaluation of transctript expression levesl by quantitative real-time PCR. The effect of each estrogen on transctript expression was evaluated by quantitative real-time PCR. The GAPDH-normalized fold changes are expressed as the mean±SD from three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (compared to control).

KNTC2 (also referred to as highly expressed in cancer 1, HEC1) is one of kinetochore protein directly involved in the kinetochore microtubule interactions, the chromosome congression, and the spindle checkpoint signaling [Martin-Lluesma *et al*., 2002; DeLuca *et al*., 2006]. High levels of KNTC2 were observed in the majority of the lung cancers of various histologic types, and the elevated expressions were associated with the poorer prognosis of the non-small cell lung carcinoma patients [Hayama *et al*., 2006]. Considering that this simultaneous up-regulation is a frequent and important feature of the cell growth and survival of the lung cancer, selective suppression of the KNTC2 activity could be a promising therapeutic target for the treatment of lung cancers. Moreover, the mitotic checkpoint is an important determinant for the efficacy of the microtubule-targeting drugs in eradicating the cancer cells [Lee *et al*., 2004]. Interestingly, the depletion of KNTC2 protein by the cytotoxic recombinant adeno-associated virus that expresses short hairpin resulted in the persistent spindle checkpoint activation, followed by the death of the tumor cells [Li *et al*., 2007].

In conclusion, *TUBA1B* and *KNTC2,* the mitogenic estrogen-dependently expressed genes, were identified in the breast cancer cells. The elucidation of the estrogen effects on the gene expression could be helpful in understanding the action mechanism of the estrogens, because the estrogens are highly involved in the progress of breast cancer. Furthermore, both genes could be promising therapeutic and prognostic biomarkers for the breast cancers.

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