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Identification and characterization of estrogen receptor variants in prostate cancer cell lines

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Abstract

A sensitive semi-nested reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was performed to evaluate estrogen receptor-α (ER-α) mRNA expression in prostate cancer cell lines. We demonstrated the presence of wild-type ER-α (wt ER-α) and five ER-α variants, designated ER-αA, B, C, D, and E. Unlike ER-αA and D, ER-αB, C, and E were not previously reported in normal or cancerous mammalian cells. DNA sequencing analysis of these ER-α variants revealed the genetic changes to be either in-frame or out-of-frame deletions. The expression of each ER-α variant differs significantly depending on the androgen responsiveness, tumorigenic and metastatic potentials of each prostate cancer cell line. The potential functional significance of ER-α variants was assessed in yeast two-hybrid and ERE promoter-reporter mammalian transcription assay systems. The results of these studies indicated that none of the ER-α variants can form homo- or heterodimers either with wt ER-α or among themselves in vivo, and that these ER-α variants have no demonstrable transcriptional or dominant-negative activity, as assessed in vitro.

1. Introduction

One of the standard therapies for the treatment of prostate cancer is the use of estrogen agonists, such as diethylstilbestrol, which presumably block endogenous testicular androgen production and produce medical castration [1]. Estrogen also appears to directly act on prostate cells by inducing dramatic histomorphological changes, such as hyperplastic and dysplastic changes, and marked inhibition of growth, when applied to either developing or adult hosts [2,3]. In cell or organ cultures in vitro, estrogen was demonstrated to elicit direct action on the growth of prostate cells and affect estrogen receptor (ER)-mediated reporter gene transcription [4–7]. Despite direct and indirect interaction between estrogen and prostate growth and gene expression, the status of ER in normal prostate development and cancer progression is controversial and is the subject of recent intense investigations [8–13].

There are presently two broad categories of ERs in the prostate gland, ER-α and ER-β [14]. ERs share common domain structures with the steroid hormone receptor superfamily, in which discreet regions are responsible for ligand binding, DNA binding, dimerization, nucleolocalization, and transcriptional activation [15–17]. Among the members of the ER family, the structure and function of ER-α have been well documented at the genetic and molecular levels. The heterogeneity of ER-α was demonstrated in estrogen-responsive normal and unresponsive normal and malignant tissues [16]. Because of the potential importance of ERs in mediating the direct action of estrogen in regulating prostate cell growth and gene transcription and the controversial evidence in the literature supporting the presence of ER-α in prostate cancer cells, we determined the presence of ER isoforms in human prostate cancer cell lines with defined androgen-dependent and tumorigenic and metastatic potentials [18]. In this report, we provide evidence that ER-α and its variants were present in prostate cancer cell lines. Differences among ER-α variants were found in

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prostate cancer cell lines with differing androgen dependency and tumorigenic and metastatic phenotypes. Novel forms of ER-α variants were detected in a human prostate cancer cell line, ARCaP, which exhibits androgen- and estrogen-inhibited phenotypes with marked enhanced malignant potential, including growth and metastasis to the skeleton [19]. Interestingly, despite the presence of ER-α variants in prostate cancer cell lines, no demonstrable activity was found between ER-α variants and wild-type ER-α in both yeast two-hybrid and mammalian transcription assay systems.

2. Materials and methods

2.1. Cell culture

Both human prostate cancer cell lines, LNCaP, C4-2 and ARCaP and an ER-α positive human breast cancer cell line, MCF-7, were routinely cultured in T medium (Life Technologies, Gaithersburg, MD) containing 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. The sources and derivation of these prostate cancer cell lines were reported previously [19,20]. Cells with several different passage numbers were used in our experiments (LNCaP: 25, 30, 35 and 54; C4-2: 18, 28 and 30; ARCaP: 14 and 25; MCF-7: 60).

2.2. RNA preparation

Total RNA was extracted from the LNCaP, C4-2 and ARCaP cells according to the manufacturer's protocol (Biotecx Laboratories, Houston, TX). Briefly, 0.2 ml of RNAzol ™ B per 10^6 cells was added to the cultured cells after removing the culture media. RNA was isolated by passing the cell lysate several times through a pipette, extracted by adding 0.1 vol chloroform to 1 vol homogenate, precipitated by adding 1 vol isopropanol to 1 vol aqueous phase, washed with 75% ethanol, and dissolved in RNase-free water.

2.3. RT-PCR

All primers used in the experiments are listed in Table 1, with their locations and orientations shown in Fig. 2a.

First strand cDNA was synthesized from 1.0 μg of total celluar RNA with the oligoprimer P0 specific for the ER gene using M-MLV reverse transcriptase as recommended by the supplier (Perkin Elmer Corp., Norwalk, CT) in a total volume of 20 μl. One microliter of the first strand cDNA synthesis reaction mixture was used for a semi-nested PCR procedure involving two rounds of amplification of the ER-α cDNA in a final volume of 50 μl. Primers P1 and P2 were used in the first round amplification. One microliter of the amplified product was used in the second round with primers P1 and P3 as previously described [21]. In both rounds, the PCR reaction mixture without cDNA template consisted of the following components: 10 pmol of each primer, 200 μmol/l of each dNTP, 1.5 mmol/l $MgCl_2$, 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl and 1.25 U of Taq polymerase in a final volume of 50 μl. All PCR amplifications were performed for 35 cycles using the following cycling parameters: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. This was preceded by an initial denaturation step at 94°C for 2 min. The final primer extension step was carried out at 72°C for 7 min.

Table 1
Primers used in the experiments

Primers	Nucleotide composition (5′→3′)	Nucleotide positions (bp)[a]	Remarks
P0	GAGGGTAAAATGCAGCAGGGATTATCTGAACCG	2040–2072	Antisense
P1	TGCCCTACTACCTGGAGAACG	615–635	Sense
P2	CGGGATCCTCAGACTGTGGCAGGGAA	2003–2020	Antisense; containing a BamHI site
P3	GCCTCCCCCGTGATGTAA	1978–1995	Antisense
P4	CTTCTGCCCTGCGGGGACACGGTCTGCAC	184–212	Sense
P5	TGGTCAGTAAGCCCATCATCG	1254–1274	Antisense
P6	CGGGTACCATGACCATGACCCTCCAC	231–250	Sense; containing a KpnI site
P7	CGAAGCTTCACTGAAGGGTCTG	1234–1255	Antisense
P8	CGGAATTCTCTAAGAAGAACAGCCTGG	1133–1151	Sense; containing an EcoRI site
P9	CGGAATTCGCCAACCTGGCAGAC	1272–1285	Sense; containing an EcoRI site
P10	CGGAATTCGGCTTTGTGGATTTGAC	1328–1344	Sense; containing an EcoRI site
P11	CGGGATCCTCAGACTGTGGCAGGGAAACCCTCTGCCTCCCCCG TGATG	1981–2020	Antisense; containing a BamHI site
P12	CGGGTACCATGACCATGACCCTCCAC	230–250	Sense; containing a KpnI site
P13	CGATTATCTGAATTTGGCCTG	682–702	Antisense

[a] Nucleotide positions are shown as previously defined [23].

To extend the analysis of exon 1, we used P4, P5, P6, and P7 primers to perform a nested PCR amplification, with P4 and P5 primers used in the first round amplification and P6 and P7 primers used in the second round amplification, as described above.

2.4. Southern blot

The RT-PCR products were separated on a 0.7% agarose gel and transferred to a Zeta membrane (Bio-Rad Laboratories, INC., Richmond, CA). An approx. 1.4 kb ER-α cDNA was amplified by PCR with the P2 and P3 primers using an ER-α plasmid as a template (kindly provided by Dr Ming-Jer Tsai from the Baylor College of Medicine, Houston, TX). The cDNA fragment was labelled with digoxigenin using DIG High Prime Labelling and Detection Starter Kit I according to the supplier's instructions (Roche, Indianapolis, IN). Hybridization was carried out overnight at 42°C in a DIG Easy Hyb solution with 50% formamide. Following washing of the membranes, the hybridized probes were immunodetected with anti-digoxigenin-alkaline phosphatase, Fab fragment, and visualized with the colorimetric substrates nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP).

2.5. Cloning and sequencing of RT-PCR products

The RT-PCR products were purified from a 0.7% agarose gel using the Wizard PCR Preps DNA Purification System Kit (Promega Corp., Madison, WI) and ligated to the pGEM-T Easy vector (Promega). The ligation mixture was transformed into *Escherichia coli* DH5α. The resulting positive clones were sequenced by the standard dideoxynucleotide chain termination method using an automatic sequencer by the University of Virginia Macromolecular Core Facility.

2.6. Plasmid construction for yeast two-hybrid system

All cDNA fragments containing the C-terminal ligand-binding domain (LBD) of the wt ER-α and its variants were obtained by PCR amplification using the recombinant pGEM-T Easy vectors as templates. The upstream primer used for wt ER-α and ER-αA variant was P8. The upstream primers used for ER-αB and ER-αD variants were P9 and P10, respectively. The downstream primer used was P11. The PCR products were cloned into an EcoRI-BamHI digested pGBKT7 vector (Clontech Laboratories, Palo Alto, CA) for the construction of bait vectors and cloned into an EcoRI-BamHI digested pGADT7 vector (Clontech) for the construction of prey vectors. The bait sequences were fused with GAL4 DNA-binding domain (DBD) sequence and the prey sequences fused with GAL4 transcription activation domain (TAD) sequence. Each GAL4 DBD and GAL4 TAD fusion cDNA construct was partially sequenced to confirm a correct reading frame.

2.7. Plasmid construction for mammalian expression system

We used recombinant PCR to amplify the full-length wt ER-α and its variants [22]. Initially, using primers P12 and P13 and the RT-PCR products amplified by the P4, P5, P6, and P7 primers as a template, we amplified an approx. 470-bp fragment A. Similarly, using primers P1 and P11 and the respective RT-PCR products amplified by the P1, P2, and P3 primers as a template, we amplified B fragments in various lengths due to the existence of the multiple ER variants. Using the isolated fragments A and B, which had an overlapping region of 88 bp (bases 615-702) and primers 11 and 12, we amplified the full-length wt ER-α and the its variant cDNAs. The cDNA fragments were cloned into a KpnI-BamHI digested pcDNA3 (Invitrogen Corp., Carlsbad, CA) vector. The cDNA constructs were partially sequenced to confirm a correct ER-α reading frame.

2.8. In vitro translation

^{35}S-labeled proteins were generated with the TNT Quick Coupled Transcription/Translation Systems (Promega) according to the manufacturer's instructions, using the pcDNA3 vectors containing either the wt ER-α or its ER-α variants as templates. Briefly, the reaction components were assembled in a final volume of 50 μl that consist of 40 μl TNT Quick Master Mix, 2 μl [^{35}S] methionine (10 μCi/μl, 1000 Ci/mmol), and 1 μg DNA template. The reaction was incubated at 30°C for 90 min. Three microliters of the translation products was removed and analyzed by SDS-PAGE under a denaturing and reducing condition.

2.9. Assessment of dimerization of the ligand-binding domains of the ER-α variants in yeast

The yeast strain HF7C was transformed with the bait and prey plasmids according to the manufacturer's instructions (Clontech). Transformants were selected for growth on synthetic medium lacking tryptophan and leucine, and transferred to Whatman No. 5 filters. The yeast colonies on the filters were cultured overnight on selection medium with 1 μM 17β-estradiol without adding histidine, tryptophan, and leucine. The fresh colonies were then assayed for activation of the lacZ reporter using the β-galactosidase colony-lift filter assay.

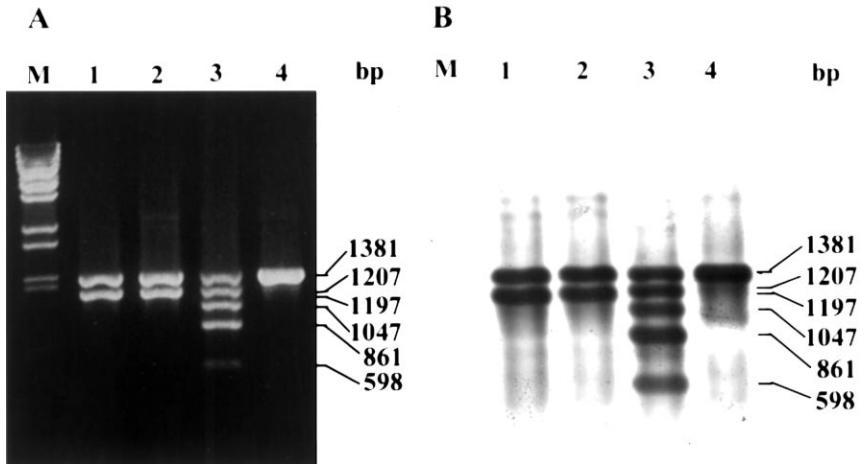

Fig. 1. Detection of the ER-α variants in prostate cancer cell lines. (A) Typical results of agarose gel electrophoresis of RT-PCR amplification products when using the semi-nested primers P1, P2 and P3. (B) Southern blot analysis of RT-PCR amplification products. Lane M: molecular weight markers of λDNA digested with BstEII; Lane 1, LNCaP cells; Lane 2, C4-2 cells; Lane 3, ARCaP cells; Lane 4, positive control with the plasmid containing the wt ER-α cDNA as a template.

2.10. Transient transfection assays

LNCaP and MCF7 cells were maintained in T-media supplemented with 5% and 10% FBS, respectively, in the 37°C incubator containing 5% CO_2. Twenty-four hours before transfections, 1.6×10^5 LNCaP cells per well were plated in 12-well dishes in T-media containing 5% FBS and 3.2×10^5 MCF7 cells per well were seeded in 6-well plates in T-media containing 10% FBS. Cells were transfected with the wt ER-α vector and/or its variant vectors as well as ERE-tk-luciferase reporter construct (kindly provided by Dr Ming-Jer Tsai, Baylor College of Medicine, Houston, TX) in RPMI 1640 phenol red-free media using Lipofectamine Plus reagent (Life Technologies). pCMV-β-gal was used as an internal control. Four hours later, transfection media was completely removed, and cells were further incubated in RPMI 1640 phenol red-free media containing 5% DCC (dextran-coated charcoal-treated serum) with 5 nM 17β-estradiol or vehicle (0.01% ethanol). For transcriptional activity analysis, the cells were washed with PBS and harvested 36 h after transfection. Cell extracts were assayed for luciferase activity using the luciferase assay system (Promega) and the values were normalized by the internal control β-galactosidase activity. Data are presented as the mean (± SD) of triplicate values obtained from representative experiments that were independently repeated at least two times.

3. Results

3.1. Assessment of the presence of wt ER-α and its variants in prostate cancer cell lines by semi-nested RT-PCR

The expression of ER-α in prostate cancer is controversial possibly due to the low level of ER-α expression [8–13]. To improve sensitivity and specificity, we performed a semi-nested RT-PCR analysis of ER-α and its variants in prostate cancer cell lines using the semi-nested primers P1 (exon 1), P2 (exon 8), and P3 (exon 8), as described in Section 2. As shown in Fig. 1(a), all tested prostate cancer cell lines (LNCaP, C4-2, and ARCaP) revealed readily detectable bands that represent wt ER-α and its variants. In LNCaP and C4-2 cells, we detected two clear bands estimated at 1381 (wt ER-α) and 1197 (ER-αA) bp. In ARCaP cells, however, we detected multiple bands estimated at 1381 (wt ER-α), 1207 (ER-αB), 1047 (ER-αC), 861 (ER-αD), and 598 (ER-αE) bp. The relationship of these bands to the wt ER-α cDNA sequence was confirmed by Southern blot with 1381 bp of the wt ER-α probe [Fig. 1(B)]. We repeated these experiments using cells with different passage numbers and evaluated ER-α and its variants by semi-nested RT-PCR. The results indicated that, with minor differences in the relative ratios of wt ER-α and its variants, the transcript pattern was similar among different cell passages (data not shown).

The amplified 1381 bp ER-α coding region from exons 1 to 8 is known to miss a 381 bp cDNA fragment within exon 1 [23]. To further determine the potential structural variations of wt ER-α and its variants in exon 1, we used the P4, P5, P6, and P7 primers to perform additional nested PCR. The result failed to reveal any deletion or mutation in exon 1 in LNCaP, C4-2, or ARCaP cells (data not shown).

3.2. DNA sequencing analysis of the ER-α variants

The RT-PCR products were cloned and subjected to DNA sequencing analysis. In addition to wt ER-α [23],

a number of exon-deleted ER-α variants were identified (Fig. 2). The ER-αA transcript was found to correspond to an ER-α variant precisely deleted in exon 7 as previously reported [24,25]. The predicted protein is identical to the wt ER-α protein up to amino acid 456, followed by 10 novel amino acids that would therefore delete a portion of the LBD of the wt ER-α receptor. DNA sequencing analyses of ER-αB, C, and E revealed them to be novel ER-α variants. The ER-αB transcript contains a 174 bp in-frame deletion from nucleotides 1103 to 1277 within exon 4 and would encode a truncated protein of 537 amino acids missing the C-terminal LBD. The ER-αC variant showed precise deletions in exon 2 and a partial deletion from nucleotides 1082 to 1201 within exon 4, plus a 2 bp (TT) insertion in the deleted region within exon 4 and partial deletion from nucleotides 1601 to 1622 within exon 7. The putative ER-αC protein is identical to the wt ER-α up to amino acid 151 followed by one novel amino acid, and missed the entire DNA-binding domain (DBD), hinge region, and LBD. The ER-αE transcript has deletions of exons 2 to 6 from nucleotides 698 to 1503 that resulted in a frame shift. The predicted protein is therefore identical to wt ER-α up to amino acid residue 155 followed by 30 novel amino acids, and missed the entire DBD, hinge region, and LBD. The ER-αD variant represents an ER mRNA variant precisely deleted in both exons 4 and 7. This deletion resulted in an ER- αD transcript, which contains an in-frame deletion and would encode a protein of 363 amino acids deleted in the hinge region and part of the LBD of the wt ER-α receptor. The structures of both ER-αD and ER-αA variants were

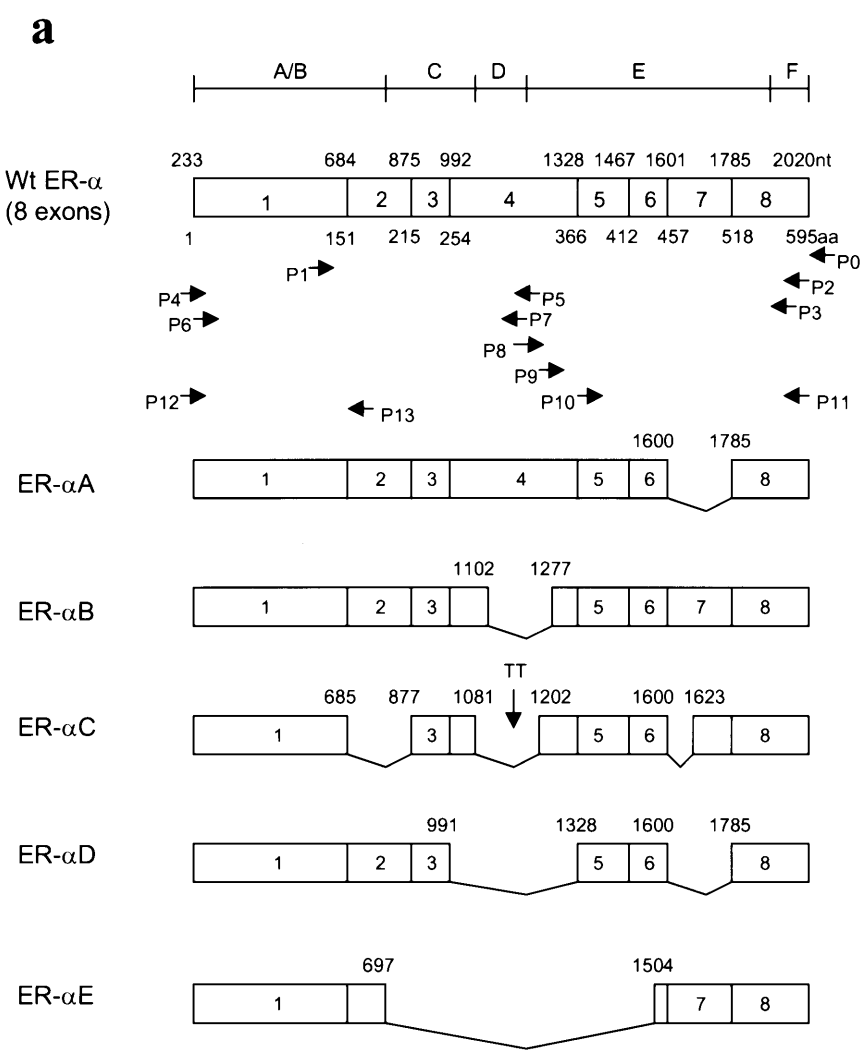

Fig. 2. Structure of the ER-α variants. (a) Schematic representation of the wt ER-α and its ER-α variant cDNAs. Funtional regions of the wt ER include the amino-terminal transactivation domain A/B, the DNA-binding domain C, the hinge region D, the ligand-binding domain E, and the carboxy-terminus F. Numbers on the top of the boxes indicate nucleotide positions as previously defined [23] except for the substitution of G for T at nucleotide 1431. Numbers below the boxes refer to positions of amino acids. Arrows indicate the positions of primers used for RT-PCR amplification. (b) Nucleotides and codon translation of junction region sequences of the ER-α variants, with the amino acid numbers as previously described [23] shown below the amino acids. The junction is indicated by double oblique lines. Stop codons are shown by "*".

b

ER-αA

```
          Exon 6          exon 8
...CTT AAT TCT // GTA ACA AAG GCA TGG AGC ATC TGT ACA GCA TGA...
... Leu Asn Ser    Val Thr Lys Ala  Trp Ser  Ile Cys Thr  Ala   *
               456
```

ER-αB

```
              exon 4          exon 4
...GCT GCC AAC // CTG GCA GAC...
...Ala  Ala  Asn    Leu  Ala  Asp...
             290    349
```

ER-αC

```
          exon 1          exon 3              exon 4                exon 4
...TTC TAC AGG // ACA TAA CGA.....GGG TCT GCT // TT // CCC CCC ATA.....CTT
  Phe Tyr  Arg    Thr   *
           151

  exon 7      exon 7
  AAT TCT // GCA CCC TGA...
```

ER-αD

```
          exon 3          exon 5              exon 6          exon 8
...ATG AAA GGT // GGC TTT GTG.....CTT AAT TCT // GTA ACA AAG GCA TGG
  Met  Lys  Gly    Gly  Phe Val      Leu Asn Ser    Val  Thr  Lys  Ala  Trp
            253    366

  AGC ATC TGT ACA GCA TGA...
  Ser  Ile  Cys Thr  Ala   *
```

ER-αE

```
          exon 2                exon 6
...AAT TCA GAT // CTT CGA CAT GCT GCT GGC TAC ATC ATC TCG GTT CCG CAT
  Asn Ser  Asp   Leu Arg  His  Ala  Ala  Gly  Tyr  Ile  Ile   Ser  Val  Pro  His
           155

  GAT GAA TCT GCA GGG AGA GGA GTT TGT GTG CCT CAA ATC TAT TAT TTT
  Asp  Glu  Ser Ala  Gly  Arg  Gly  Val  Cys Val  Pro  Gln  Ile  Tyr  Tyr  Phe

  GCT TAA...
  Ala   *
```

Fig. 2. (*Continued*)

reported in normal and neoplastic human breast cells and tissues [24–27].

3.3. Analyses of dimerization of the LBD of the ER-α variants in yeast

Since all of the ER-α variants have deletions at the C-terminal LBD, which is required for dimerization [28–31], we used a yeast two-hybrid system to investigate the dimerization of the LBD of wt ER-α and its variants. As shown in Table 2, neither GAL 4 DBD wt ER-α nor GAL 4 TAD wt ER- α alone can activate the transcription of the lacZ reporter gene with or without the addition of 17 β-estradiol, which is in line with previous work [32,33]. The transcription, however, could be activated when Gal 4 DBD wt ER-α and Gal 4 TAD wt ER-α were co-expressed in yeast in the presence of 17 β-estradiol, indicating that the interaction between wt ER-α LBD in yeast is 17 β-estradiol-dependent.

We then evaluated the potential interaction of ER-α variant LBDs with the wt ER-α LBD in yeast in the presence or absence of 17 β-estradiol. The result showed that all of the ER-α variant LBDs failed to interact with wt ER-α LBD, either in the presence or absence of 17 β-estradiol. The result also showed that no interaction among ER-α variant LBDs, either as hetero- or homodimers, occured in the yeast assay system. Considering the possible conformational changes resulting from the fusion expression of the ER-α LBDs with GAL4 DBD or Gal 4 TAD, we switched GAL4 cloning vectors by expressing the ER-α variant LBDs from GAL4 TAD to GAL4 DBD. The results remained unchanged (Table 2).

3.4. Assessment of transcriptional activity of the ER-α variants in mammalian cells

To determine whether all ER-α variants constructed in the pCDNA3 vector can be expressed, we used in

Table 2
Characterization of dimerization of the ER variant ligand-binding domains ×

Transforming plasmids		β-galactosidase activity	
Bait	Prey	No treatment	1 μM 17β-estradiol
GAL4 DBD-wtER-α		–	–
	GAL4 TAD-wt ER-α	–	–
GAL4 DBD-wtER-α	GAL4 TAD	–	–
GAL4 DBD	GAL4 AD-wt ER-α	–	–
GAL4 DBD-wt ER-α	GAL4 AD-wt ER-α	–	+
GAL4 DBD-wt ER-α	GAL4 TAD-ER-αA	–	–
GAL4 DBD-wt ER-α	GAL4 TAD-ER-αB	–	–
GAL4 DBD-wt ER-α	GAL4 TAD-ER-αD	–	–
GAL4 DBD-ER-αA	GAL4 TAD-ER-αA	–	–
GAL4 DBD-ER-αA	GAL4 TAD-ER-αB	–	–
GAL4 DBD-ER-αA	GAL4 TAD-ER-αD	–	–
GAL4 DBD-ER-αB	GAL4 TAD-ER-αB	–	–
GAL4 DBD-ER-αB	GAL4 TAD-ER-αD	–	–
GAL4 DBD-ER-αD	GAL4 TAD-ER-αD	–	–
GAL4 DBD-ER-αA	GAL4 AD-wt ER-α	–	–
GAL4 DBD-ER-αB	GAL4 AD-wt ER-α	–	–
GAL4 DBD-ER-αD	GAL4 TAD-wt ER-α	–	–
GAL4 DBD-ER-αB	GAL4 TAD-ER-αA	–	–
GAL4 DBD-ER-αD	GAL4 TAD-ER-αA	–	–
GAL4 DBD-ER-αD	GAL4 TAD-ER-αB	–	–

vitro transcription and translation systems to synthesize the ER-α variants. As shown in Fig. 3, all ER-α variants are expressed efficiently. To examine whether ER-α variants may have transcriptional activity on the target gene, we transfected the wt ER-α (positive control) or its variant into LNCaP cells along with the ERE-tk-luciferase reporter construct. Results demonstrated that, upon addition of 17β-estradiol, only the wt ER-α, but not its variants enhanced the luciferase reporter gene transcription in LNCaP cells (Fig. 4). We then evaluated the ability of the ER-α variants to serve as a co-activator or a dominant negative factor that participate in the wt ER-α activated ERE-luciferase gene transcription. Fig. 5(a) and (b) shows that the ER-α variants neither significantly enhanced nor inactivated the wt ER-α mediated ERE-luciferase reporter gene activity in LNCaP or MCF7 cells, respectively, in response to 17β-estradiol induction. Taken together, our results demonstrated that none of the ER-α variants is transcriptionally active on the ERE-tk-luciferase reporter gene construct in the prostate and breast cancer cell lines tested.

4. Discussion

We employed a sensitive nested RT-PCR amplification to detect the presence of wt ER-α as well as its variants in prostate cancer cell lines with differing androgen sensitivity, tumorigenicity, and metastatic potentials [18]. We confirmed the presence of two previously described ER-α variants, exon 7 and exons 4 and 7-deleted ER-α variants (ER-αA and ER-αD). Both of them have been shown to be expressed in human breast tissues and human breast cancer cell lines. To our knowledge, however, there is no report on exon 7-deleted ER variant identified in prostate cancer cells. In addition, we identified three novel ER-α variants, ER-αB, ER-αC and ER-αE. Unlike ER-αA and ER-αD, ER-αB, -αC and –αE contain intra-exonic deletions at exons 4, 4/7, and 2/6, respectively. The simplest interpretation of the precise deletions of exon 7 only, or both exons 4 and 7 is that these deletions resulted from alternative or inaccurate splicing of an original wt ER-α mRNA transcript. Three novel ER-α variants, however, could arise due to abberant splicing, which did not

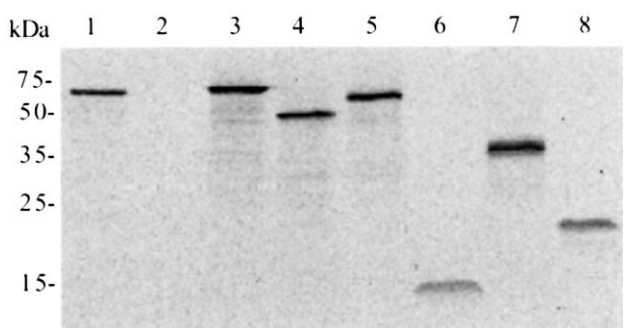

Fig. 3. in vitro translation of ER-α variants. Lane 1, luciferase control DNA; Lane 2, pcNA3; Lanes 3–8, the pcNA3 vectors containing wt ER-α, ER-αA, B, C, D and E, respectively. The molecular weight of wt ER-α is 65 kDa. The estimated molecular weights of ER-αA, B, C, D and E are 51, 59, 17, 40 and 20 kDas, respectively.

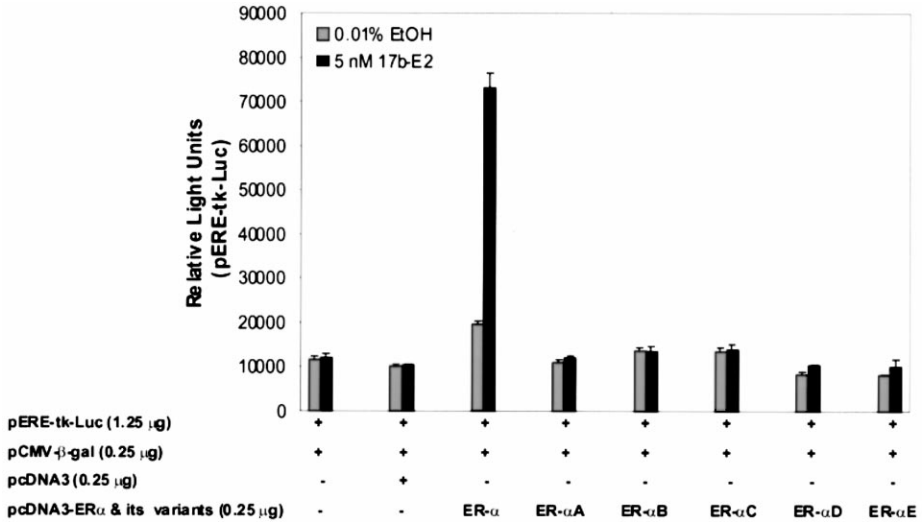

Fig. 4. Human ER-α isoforms mediated ERE-tk-Luc activities in the prostate cancer cell line. LNCaP Cells were cultured in 12-well culture plates and cotransfected with the respective ER-α variant (0.25 μg/well) and ERE-tk-Luc (1.25 μg/well) along with the β-gal as an internal transfection efficiency control (0.25 μg/well). After 36-h incubation with 5 nM 17β-estradiol or with 0.01% ethanol vehicle, luciferase activity was measured as luciferase units and corrected by internal control β-gal activity. Data are represented as the mean (±SD) of triplicate values.

conform to the consensus donor and acceptor splice signals of GT/AG. The RT-PCR, southern blot and sequencing showed that the pattern of ER-α mRNA expression in ARCaP cells is different from that of LNCaP and C4-2 cells. This difference may explain the phenomenon that estrogen stimulated the growth of LNCaP and C4-2 cells but inhibited that of ARCaP cells [19]. The relative ratio of the wt ER-α to its ER-α variants in ARCaP cells is lower than that in LNCaP and C4-2 cells, suggesting that the wt ER-α and increased ER-α variants in ARCaP cells may be responsible for the differential actions of estrogen in human prostate cancer cell lines.

Although the amount of mRNAs of the wt ER-α and its variants could be too low to be detected by Northern blot analysis in human prostate cancer cell lines (data not shown), it is conceivable that the wt ER-α and its variants could be transcribed and translated in intact cells. This speculation may be supported by the fact that in an in vitro transcription and translation system, the wt ER-α and its variants were readily detectable by SDS-PAGE analysis. We cannot exclude the possibility that the wt ER-α and its ER-α variants might have been detected using 50 μg of total cellular proteins by Western blot analysis (data not shown). Considering the specificity of the ER-α antibody (sc-7207, Santa Cruz Biotechnology, Inc.) we used, it is hard to precisely determine the presence of the wt ER-α and its variants.

Interaction of the ER-α with its ligands is thought to be mediated by a C-terminal LBD, whose dimerization is part of its normal functioning [34]. It has been reported that specific point mutations introduced at residues R-507, L-511 and I-518 of the mouse ER-α receptor (corresponding to R-503, L-507 and I-514 of the human ER-α receptor, respectively) prevent efficient receptor dimerization, and inhibit the binding of an ERE [29]. In our experiments, analysis of the possible functional role of these ER-α variants in yeast assay system revealed that the LBDs of these ER-α variants neither formed homo- nor heterodimers. The predicted ER-αC and ER-αE proteins do not contain the DBD and LBD. The putative ER-αA, ER-αB and ER-αD proteins have deletions from amino acids 457-553, 302-348 and 302-364, respectively, suggesting that these regions are important for the dimerization of LBD. The inability to form the dimerization may also explain the fact that these ER-α variants have no demonstrable transcriptional or dominant-negative activity in a mammalian cell assay system. These results are consistent with some of the previous reports, where a number of ER-α variants failed to interfere with wild-type ER-α activity in breast cancer cell lines, yeasts, and other systems [16]. There are a few reports, however, indicating that ER-α variants may serve as a dominant-negative factor in blocking wt ER-α function in gene transcription [16,25,35].

ER-α variants, however, may have other unsuspected functions, as discussed below. (1) ER-α variants may interact with other transcriptional regulators in affecting downstream ER-α-mediated target gene expression. (2) The interaction between ER-α variants with other transcriptional factors can occur at the level of either protein-protein interaction or protein-RNA interaction [36,37]. (3) The activity of ER-α variants and their interactions with wt ER-α or other transcriptional factors may be highly dependent on cell background and growth conditions. It has been reported that ER-α

variants may serve as dominant-negative factors for wt ER-α in yeast cells but not in Hela cells [25]. In this context, it is interesting to note that the pattern of ER-α variants is identical between LNCaP and its lineage-derived C4-2 cells, while different profiles of ER-α variants were detected in ARCaP cells, which were derived from a different patient [19]. (4) Although the expression level of both wt ER-α and its variants in prostate cancer cell lines is low, they could serve as important functional regulatory molecules by interacting with other protein factors and modulating the levels of critical gene expression. Moreover, it is possible that under physiological conditions, wt ER-α and its variants may interact with each other or with other undefined transcriptional regulators that culminate in a directed gene expression profile and in a highly spatial and temporal relationship during normal development and neoplastic progression. The potential role of ER-α

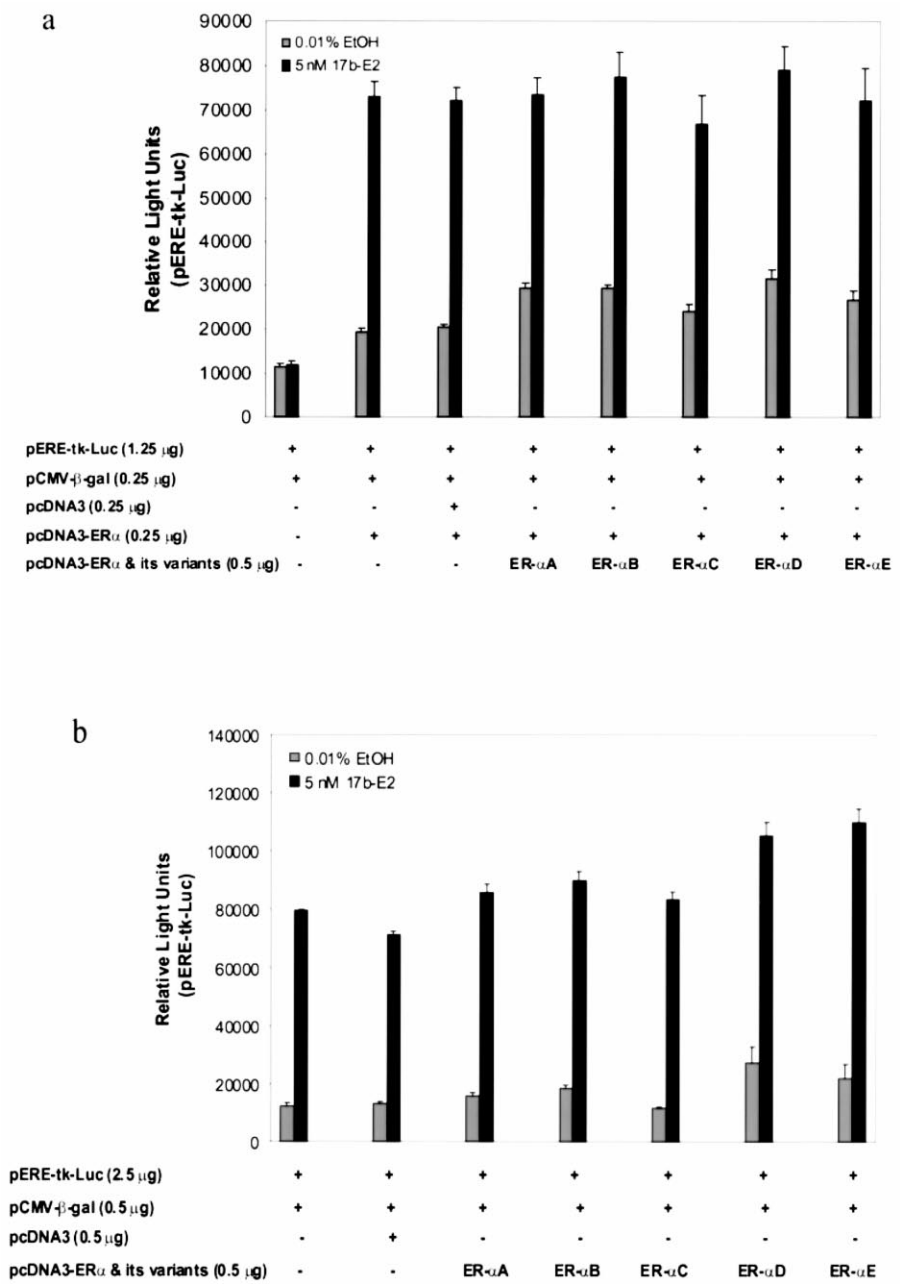

Fig. 5. (a) The wt ER-α mediated ERE-tk-Luc activity was not affected by the ER-α variants in LNCaP cells. Cells were cultured in 12-well culture plates and cotransfected with the wt ER-α (0.25 μg/well) and respective ER-α variants (0.5 μg/well) along with ERE-tk-Luc (1.25 μg/well) reporter construct and β-gal internal control (0.25 μg/well). Data are represented as the mean (±SD) of triplicate values. (b) None of the ER-α variants has significant influence on the transcriptional activity of ER in MCF7 cells. For this assay, cells were cultured in 6-well culture plates and cotransfected with respective ER-α variants (0.5 μg/well) along with ERE-tk-Luc (2.5 μg/well) and β-gal internal control (0.5 μg/well). Data are represented as the mean (±SD) of triplicate values.

variants interacting with wt ER-α or other transcriptional factors might be an attractive future focus to define hormonal sensitivity of normal and neoplastic prostate and breast growth in situ.

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