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Estrogen receptor β 2 and β 5 are associated with poor prognosis in prostate cancer, and promote cancer cell migration and invasion

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Abstract

Estrogens play a pivotal role in the development and progression of prostate cancer (PCa). Their actions are mediated by estrogen receptors (ERs), particularly ER β in the prostate epithelium. With the discovery of ER β isoforms, data from previous studies that focused principally on the wild-type ER β (ER β 1) may not be adequate in explaining the still controversial role of ER β (s) in prostate carcinogenesis. In this study, using newly generated isoform-specific antibodies, immunohistochemistry (IHC) was performed on a tumor microarray comprised of 144 specimens. IHC results were correlated with pathological and clinical follow-up data to delineate the distinct roles of ER β 1, ER β 2, and ER β 5 in PCa. ER β 2 was commonly found in the cytoplasm and was the most abundant isoform followed by ER β 1 localized predominantly in the nucleus, and ER β 5 was primarily located in the cytoplasm. Logistic regression analyses demonstrated that nuclear ER β 2 (nER β 2) is an independent prognostic marker for prostate specific antigen (PSA) failure and postoperative metastasis (POM). In a Kaplan–Meier analysis, the combined expression of both nER β 2 and cytoplasmic ER β 5 identified a group of patients with the shortest POM-free survival. Cox proportional hazard models revealed that nER β 2 predicted shorter time to POM. In concordance with IHC data, stable, ectopic expression of ER β 2 or ER β 5 enhanced PCa cell invasiveness but only PCa cells expressing ER β 5 exhibited augmented cell migration. This is the first study to uncover a metastasis-promoting role of ER β 2 and ER β 5 in PCa, and show that the two isoforms, singularly and conjointly, have prognostic values for PCa progression. These findings may aid future clinical management of PCa.

Endocrine-Related Cancer (2010) 17 675–689

Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of death among American men (Jemal *et al.* 2009). Although androgen deprivation therapies (ADTs) remain as the mainstay treatment for advanced PCas, these therapies eventually fail, in part, due to the development of androgen hypersensitivity, ligand-independent

androgen receptor (AR) transactivation, and AR gene mutations and/or amplification in PCas (Scher *et al.* 2004, Culig & Bartsch 2006). The recently discovered AR spliced variants in PCa further raise concerns on the efficacy of these therapies (Dehm *et al.* 2008, Guo *et al.* 2009). Thus, limitations revolving around ADTs have prompted exploration of other therapeutic options. Transdermal estrogen patches as a first-line

therapy in patients with locally advanced or metastatic PCa (Langley *et al.* 2008) are now in phase II trials. However, despite significant research efforts, the role(s) of estrogen in the pathogenesis of PCa remains poorly understood.

The incidence of PCa rises dramatically in men older than 55. This phenomenon could be due to hormonal changes during aging. Among older men, androgen levels drop significantly, whereas their estrogen levels remain unchanged or increased, making the estrogen to androgen ratio elevated in the aging prostate (Ho *et al.* 2006). Epidemiological studies indicate a correlation between PCa and circulating estrogen levels, but not circulating testosterone levels, among different ethnic/racial groups with diverse PCa incidence (Rohmann *et al.* 2007). In addition, estrogen is produced locally by aromatase, which converts androgen into estrogen in the prostate (Stone *et al.* 1986, Matzkin & Soloway 1992, Ellem *et al.* 2004, Takase *et al.* 2006). Estrogens are essential in supporting the normal functioning of the prostate, and yet have long been suspected as a risk factor for PCa. Long-term exposure to elevated levels of estrogen against a normal androgen background induced a high incidence of PCa in rodents (Leav *et al.* 1988, Prins *et al.* 2007, Rieke *et al.* 2008). Also, *in utero* exposure to higher levels of maternal estrogen in African-American men may be associated with higher PCa risk (Henderson *et al.* 1988), a thesis supported by animal studies (Prins 1997, Ho *et al.* 2006, Prins & Korach 2008).

Estrogen receptors (ER), ER α and ER β , are the major mediators of estrogen signaling. Upon binding of estradiol-17 β , ER in the nucleus forms a homo-/heterodimer and binds either directly to the classical estrogen-responsive element (ERE) or indirectly to an NF κ B-, Ap1-, or Sp1-binding element via tethering with their respective transcription factors, thereby initiating downstream signaling cascades (Heldring *et al.* 2007). The roles of ERs in the prostate are not fully understood. ER α is found primarily in stromal cells of the prostate, and appears to regulate the growth and differentiation of prostatic epithelial cells in a paracrine fashion (Ellem & Risbridger 2009). In the prostatic epithelium, ER β is the predominant ER subtype (Kuiper *et al.* 1996), but its function remains controversial. Hyperplasia and dysplasia have been found in the prostates of adult ER β knockout mice (Krege *et al.* 1998, Weihua *et al.* 2001), arguing for an antiproliferative role of ER β (Morani *et al.* 2008). However, other research groups did not observe such phenotypes in their ER β knockout mice (Couse *et al.* 2000, Dupont *et al.* 2000, Antal *et al.* 2008). We and others have shown that the expression of ER β in human

prostate epithelial cells decreases as PCa develops and progresses to a higher grade (Leav *et al.* 2001, Zhu *et al.* 2004), but reappears in the lymph node and bone metastases (Lai *et al.* 2004, Zhu *et al.* 2004). The dynamic change in ER β expression is epigenetically regulated by reversible cytosine methylation of an AP2 site in a CpG island located in the ER β proximal promoter (Zhu *et al.* 2004, Zhang *et al.* 2007). These findings suggest that ER β may play a protective role during early stages of prostate carcinogenesis, but either promote metastasis or support PCa cell survival at distant sites (Leav *et al.* 2001). Apropos to the postulate that ER β promotes PCa metastasis, a few reports have demonstrated an association between ER β immunopositivity in high-grade PCa and poor relapse-free survival time (Horvath *et al.* 2001, Nanni *et al.* 2009). However, further delineation of the role of ER β (s) in PCa progression is needed.

The continuing controversy over the function of ER β s in prostate carcinogenesis could be due to variable expression levels of different ER β isoforms in benign versus malignant tissues during different stages of the process. In humans, in addition to the wild-type ER β or ER β 1, four spliced variants designated as ER β 2–5 have been identified (Moore *et al.* 1998). The ER β 2–5 isoforms share the first four functional domains with ER β 1, but each has a unique activation function 2 (AF2) domain. Since isoform-specific antibodies were unavailable until very recently, the use of various antibodies directed against different domains of the molecules might have generated immunohistochemistry (IHC) patterns that contradict each other.

No functions for ER β 2–5 isoforms in the prostate have yet been identified except in one study with 48 cases, suggesting that ER β 2 may be a poor prognostic marker for PCa (Fujimura *et al.* 2001). A breast cancer study, however, has implicated nuclear ER β 2 (nER β 2) and nuclear ER β 5 (nER β 5) to predict better prognosis (Shaaban *et al.* 2008). To clarify the role of ER β 1 and its isoforms ER β 2 and ER β 5 in PCa, we evaluated the expression patterns of these three ER β s in a set of tissue microarrays (TMAs) from a cohort of 144 patients with long follow-up using newly developed in-house isoform-specific antibodies. We found that nuclear expression of ER β 2 in PCa was an independent prognostic marker for prostate specific antigen (PSA) failure (PF), postoperative metastasis (POM), and time to POM. Furthermore, the coexpression of nER β 2 and cytoplasmic ER β 5 (cER β 5) was found associated with the worst prognosis in terms of POM-free survival time. We have further demonstrated that the stable, ectopic expression of ER β 5 in PCa cells increased cell

migration and invasiveness without affecting cell growth, while the expression of ER β 2 augmented only cell invasion. In aggregate, our data suggest that ER β isoforms have variable functions and prognostic values in PCa.

Materials and methods

Patient population

With Institutional Review Board (IRB) approval, we included 144 patients with PCa who underwent radical prostatectomy at the Massachusetts General Hospital (Boston, MA, USA) from September 1993 to March 1995 in this study. All hematoxylin–eosin (H&E)-stained sections from each case were reviewed, and the Gleason score was reassigned on the basis of the current grading recommendation provided by the International Society of Urological Pathology (Epstein *et al.* 2005). The tissue blocks containing the index PCa (tumor focus with the highest Gleason score) were selected for inclusion in the TMA. The relevant clinicopathological data collected included age; pre-operative PSA; Gleason score; American Joint Committee on Cancer (AJCC) T stage; surgical margin; and POM, follow-up of PSA recurrence, and overall survival. PF was any detectable PSA after PSA nadir post-surgery. The value was 0.5 ng/ml before 1999 and 0.2 ng/ml since 1999. POM comprised bone and lymph node metastases.

TMA construction

The TMAs were constructed using a manual tissue array instrument (Beecher Instruments, Silver Spring, MD, USA). Briefly, the H&E-stained slides were reviewed for accuracy of Gleason scores and for the availability of adequate areas of each component Gleason grade pattern; the index tumor, defined as the largest and/or highest Gleason score, was identified on the slide; and the representative highest Gleason score areas were marked. Three tissue cylinders with a diameter of 0.6 mm were punched from selected areas of each donor block and brought into a recipient paraffin block. Each block contained normal prostate tissue derived from normal peripheral zone away from the tumor and benign prostatic hyperplasia. The normal tissue serves as an internal control and a reference of staining intensity for adjacent cancer foci. After construction of the TMA blocks, H&E-stained sections were made for histological evaluation.

Antibodies

ER β 1-specific antibody (GC-17, Biogenex, San Ramon, CA, USA) recognizing amino acid residue 502–518 of the AF2 domain was developed in our laboratory, and shown to be highly specific in both western blot and IHC assays (Leav *et al.* 2001). Polyclonal monospecific antibodies specific for C-terminal peptides within ER β 2 (482-MKMETLL-PEATMEQ-495) and ER β 5 (459-LMLLSHVRHAR-YAP-472) were prepared by immunizing rabbits with the targeted peptide conjugated to BSA according to the affinity-purified package offered by New England Peptide (Gardner, MA, USA). Because we found purified IgGs of ER β 2 and ER β 5 to be unstable, we used antisera for all subsequent analyses. The expression of all isoforms in western analyses was confirmed with an N-terminal-specific antibody (H150, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Western blot analyses

To evaluate the specificity of our ER β 2 and ER β 5 antisera, we expressed various ER β isoforms (ER β 1, ER β 2, and ER β 5) in HEK293 cells and used these as reagents for testing the specificity of an antiserum. Methods for cell culture maintenance, DNA transfection, and western blot analyses were similar to those previously reported (Leung *et al.* 2006). The dilution ratio for the primary antibodies used in this study was maintained at 1:1000.

IHC analyses

The detection of ER β 1, ER β 2, or ER β 5 expression on human prostate paraffin-embedded sections was carried out according to our published protocols for ER β 1 (Leav *et al.* 2001). The optimal dilution ratio for each primary antiserum was determined empirically, and found to be 1:100 for ER β 1 and ER β 5 and 1:500 for ER β 2. These dilution ratios were used throughout our study. The specificity of ER β 2 and ER β 5 antisera was determined by neutralizing each antiserum with a 10 \times excess (by weight) of its respective targeted peptide. Peptides derived from ER β 1 (GCKSSITGSECSAEDS), ER β 3 (CSWRLFMLREAS), ER β 4 (CVRHARWGEKQ-FIHLK), and ER β 5 (CSHVRHARYAP) were also applied to the IHC studies to further evaluate the antibody specificity. The antibody–peptide precipitate was removed by centrifugation at 12 000 *g* for 10 min after overnight incubation at 4 °C. The supernatant (pre-absorbed serum) was used in parallel with the primary antiserum as a negative control to establish specificity of its respective antiserum.

In TMA studies, sections were stained according to the same IHC protocol. IHC results were scored, evaluated, and graded independently in a blinded fashion by two investigators (C-L W and S W), including one experienced urological pathologist (C-L W). Nuclear and cytoplasmic signals of each ER β isoform were examined separately. Signal intensity and percentage of signal coverage of each region were scored according to the Allred scoring system (Allred *et al.* 1993). The intensity signal was graded from 0 to 3 (0, none; 1, weak; 2, intermediate; 3, strong), and the percentage of positive tumor cells was scored from 1 to 5 (1, <1%; 2, 1–10%; 3, 10–30%; 4, 30–60%; 5, >60%). For example, if the same specimen was not stained by either of the antibodies, it will be scored as 0+1 (Allred score (Ascore)=1, i.e. negative).

Subcellular localization studies

PC3 cells (ATCC, Manassas, VA, USA) were maintained under standard conditions and cultured on coverslips. A full-length sequence of ER β isoforms 1, 2, and 5 was subcloned into pEF-yellow fluorescent protein (YFP)-C1 (Clontech) and expressed as a fusion protein with an N-terminal YFP tag in PC3 cancer cells following the Lipofectamine 2000 (Invitrogen) transfection protocol. At 48 h after transfection, cells were incubated with 250 nM Mito-tracker Red CM-H2XRos (Invitrogen) for 45 min and fixed with 4% formaldehyde for 15 min. Cells were then permeabilized with 0.2% Triton X-100 before their nuclei were counterstained with 300 nM 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) and preserved in Prolong Antifade Gold reagent (Invitrogen). Fluorescent signals were captured and evaluated with an Axiovert 200M fluorescent microscope equipped with an AxioCam MRm camera and Axiovision 4.7 software (Carl Zeiss, Thornwood, NY, USA). Optical sections were generated using ApoTome (Carl Zeiss).

Construction of PC3 cells stably expressing ER β isoforms

Full-length sequences of ER β 1, ER β 2, and ER β 5 were subcloned into a pLenti6 lentiviral vector recombined with an ubiquitin promoter by Multisite Gateway recombination reactions (Invitrogen) and transfected into 293FT cells for production of lentivirus according to the manufacturer's protocol. Lentivirus carrying the LacZ gene was used as a control. The use of the ubiquitin promoter, which is a constitutive but relatively weak promoter, minimized artifacts introduced by other virus-based promoters. The titer of each lentivirus was measured, and the multiplicity of

infection of PC3 cells was 0.7. Lentivirus-infected PC3 cells were cultured in medium supplemented with blasticidin at 8 μ g/ml and selected for 3 weeks. PC3 cells stably expressing LacZ, ER β 1, ER β 2, and ER β 5 were designated as PC3–LacZ, PC3–ER β 1, PC3–ER β 2, and PC3–ER β 5 respectively. Expression of the transgenes was confirmed by real-time PCR assays.

Cell migration and invasion assays

Cellular mobility of PC3–LacZ, PC3–ER β 1, PC3–ER β 2, and PC3–ER β 5 cells was evaluated with a wound-healing assay (Rodriguez *et al.* 2005), and their invasiveness through the extracellular matrix was determined by the BD BioCoat Matrigel Invasion Chamber method (BD Biosciences, San Jose, CA, USA) following the manufacturer's protocol.

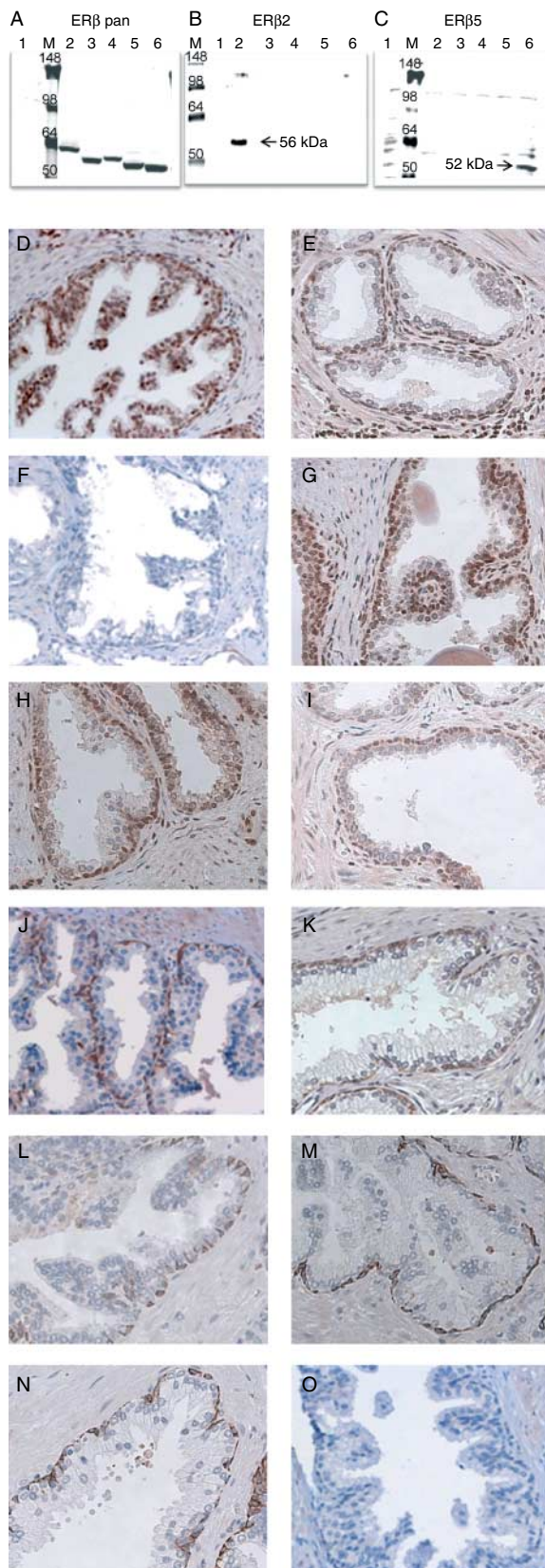
Statistical analysis

Descriptive statistics were calculated for demographic and clinical measures. Preliminary associations between Ascores measuring the expression level of cytoplasmic and nER β 1, 2, and 5 were analyzed using Mantel–Haenszel χ^2 test statistics to evaluate associations with PF and POM. A significant relationship with at least one outcome was found for one isoform of each type ($P < 0.05$). These Ascores were included in logistic regression analyses, adjusted for Gleason score and age. Ascores were dichotomized at cutpoints where slopes of Ascores changed, determined by statistical evaluations of curves fitted to the data, and visual assessment. Two cutpoints were chosen for each outcome and each Ascore. The best model was chosen by evaluating goodness-of-fit statistics. Cox proportional hazard models were used to analyze time to PF and time to POM. Kaplan–Meier plots of PF and POM survival were analyzed, stratified by dichotomized cytoplasmic and nuclear levels. An α -level of 0.05 was used to determine significance, unless otherwise stated. Analyses were performed with SAS for Windows, Version 9.1.2 (SAS Institute, Cary, NC, USA) and GraphPadPrism4 (La Jolla, CA, USA).

Results

Evaluation of ER β 2 and ER β 5 antisera for isoform specificity

A specific antibody targeting each isoform is necessary for studying the expression of ER β isoforms. An ER β 1-specific antibody (GC17) that we had raised previously has been widely used in PCa research (Leav *et al.* 2001, Adams *et al.* 2002, Pais *et al.* 2003,



Zhu *et al.* 2004). We recently also developed polyclonal monospecific antisera to ERβ2 and ERβ5 based on isoform-specific C-terminal sequences. We first overexpressed full-length ERβ1, 2, 3, 4, or 5 expression plasmids (Leung *et al.* 2006) in HEK293 cells and used the cell lysates to test the specificity of ERβ2 and ERβ5 antiserum by western blot analysis. We used a pan-ERβ antibody to confirm the expression of each ERβ isoform protein in HEK293 cells (Fig. 1A). We then reblotted the same lysates with ERβ2-specific (Fig. 1B) or ERβ5-specific (Fig. 1C) antiserum. ERβ2-specific antiserum recognized a single 56-kDa band in lane 3, which was loaded with a lysate from HEK293 cells expressing the ERβ2 transgene. No cross-reactivity with other isoforms (lanes 2, 4, 5, and 6) was detected in this ERβ2 western blot. Similarly, the ERβ5 antiserum strongly recognized a 53-kDa band in lane 6 that was loaded with a lysate from HEK293 cells harboring the ERβ5 transgene.

We then investigated whether we could use the new antisera for IHC. To validate the specificity of an antiserum, we immunostained serial prostate archival sections with or without ERβ isoform-specific peptides. At low magnification ($\times 100$), the ERβ2 antiserum primarily stained the cytoplasm of the basal and luminal epithelial cells of benign glands (Fig. 1D) and also stained the nuclei of some stromal cells. The immunopositive signals were not blocked by other ERβ isoform-specific peptides (Fig. 1E, G, H, and I) but totally abolished by the ERβ2 peptide (Fig. 1F). These results showed that the specificity of ERβ2 antiserum for ERβ2 was excellent. Similarly, ERβ5 antibody strongly stained the cytoplasm of basal epithelial cells but not the luminal epithelial cells ($\times 100$) and weakly stained some stromal cells

Figure 1 Specificity of ERβ isoform-specific antibodies. Western blot analyses: HEK293 cells expressing ERβ1–5 lysate were tested against ERβ pan antibody ((A) Santa Cruz H150), ERβ2-specific antibody (B), and ERβ5-specific antibody (C). Lane 1 was loaded with control cell lysate (HEK293 cells only), whereas lanes 2–6 were loaded with HEK293 cell lysate expressing ERβ1–5 respectively. M stands for protein marker (Invitrogen SeeBlue Plus2 protein ladder). The expected protein band of the ERβ isoforms 2 and 5 was labeled by an arrow in the middle and right panel respectively. Immunohistochemical (IHC) analyses: IHC analyses of benign prostatic tissue were carried out using ERβ2- (D–I) and β5-specific antisera (J–O). ERβ1-, ERβ2-, ERβ3-, ERβ4-, and ERβ5-specific peptides were applied to the IHC analyses. Figures showing IHC analyses with or without ERβ isoform-specific peptide are arranged as follow: D and J (no peptide); E and K (+ERβ1 peptide); F and L (+ERβ2 peptide); G and M (+ERβ3 peptide); H and N (+ERβ4 peptide); I and O (+ERβ5 peptide).

(Fig. 1J). Such immunopositive signals of ER β 5 were totally eliminated by its blocking peptide (Fig. 1O) but not by other isoform-specific peptides (Fig. 1K, L, M, and N), verifying the specificity of ER β 5 antiserum for IHC analyses.

Immunocytochemistry analyses of ER β isoforms in PCa specimens

Although we showed the antisera to be specific to their respective antigen, we also found that they may crossreact with other ER β isoforms. We therefore further examined our ER β -stained sections at high magnification ($\times 630$) to determine whether the staining pattern was unique for each antibody/antiserum. Immunostained benign glands (Fig. 2A–F) and cancer foci, consisting of Gleason grade 3 cancer glands (Fig. 2G–M), were compared in parallel. In benign prostate glands, ER β 1 was localized principally in the nuclei of basal epithelial cells (Fig. 2A and D) as previously reported for GC17 staining (Leav *et al.* 2001). ER β 1 was also localized in the nuclei of a few luminal epithelial cells but was found quite frequently in the perinuclear zone in luminal epithelial cells. The nuclei of some stromal cells were also strongly stained by the ER β 1 antibody (GC17). In contrast, the ER β 2 was localized predominantly to the cytoplasm of both basal and luminal epithelial cells, with clear localization in the supranuclear zone of the luminal cells (Fig. 2B and E); nuclear localization was uncommon. ER β 5, however, was localized almost exclusively in basal epithelial cells in benign prostate glands

(Fig. 2C and F); intracellular localization was primarily cytoplasmic. In PCa, such as in Gleason score 6 (3+3) cancer foci (Fig. 2K), some foci of ER β 1 positivity were lost in both nuclear and cytoplasmic compartments, but some remained (Fig. 2J). ER β 2 (Fig. 2H and L) and ER β 5 (Fig. 2I and M), however, displayed a diffused pattern of cytoplasmic staining, with occasional nuclear positivity. In summary, the three antibodies/antisera produced distinct staining patterns in both benign glands and cancerous prostatic foci, thereby providing support for the suggestion that each of them is highly specific and probably recognizes only one ER β isoform in IHC studies.

Subcellular localization of ER β isoforms in PCa cells

To elucidate the subcellular localization of ER β isoforms in PCa cells, we ectopically expressed ER β 1, ER β 2, or ER β 5 in PC3 cells in the form of a YFP fusion protein, with YFP serving as a control (Fig. 3A–D). YFP–ER β 1 was found principally in the nucleus, with very weak signals in the cytoplasm (Fig. 3E). The cytoplasmic signal did not overlap the Mito-tracker red signal (Fig. 3G), suggesting that ER β 1 does not reside in mitochondria in PC3 cells. Similarly, the YFP signal of ER β 2 (Fig. 3I) and ER β 5 (Fig. 3M) was found in both the nuclei and cytoplasm of the PC3 cells, and no overlapping signal with the Mito-tracker red signals was detected in both cases (Fig. 3K and O). Of interest was the stronger cytoplasmic signal of YFP–ER β 5 among the ER β isoforms.

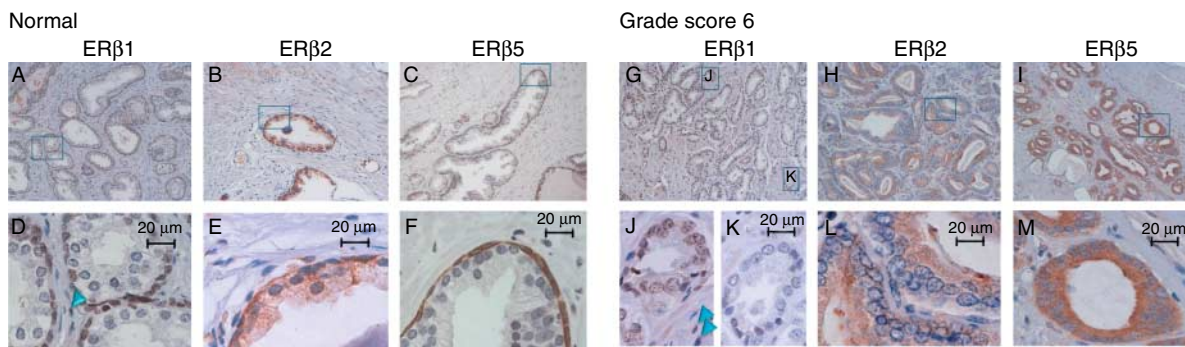


Figure 2 Immunohistochemical analyses of ER β isoforms in benign prostate glands (A–F) and prostate adenocarcinoma in PCa specimen (G–M). PCa specimens with Gleason score 6 (G–M) and its adjacent normal region (A–F) were immunostained with ER β 1-, ER β 2- and ER β 5-specific antibodies/antisera. See Materials and methods for the experimental conditions. Figures D–F and J–M are the magnified view ($\times 630$) of a region (marked by a rectangle) in Figure A–C and G–I ($\times 100$) respectively. In ER β 1 immunostaining, positive signals were found mostly in the nuclei of basal epithelial cells in benign foci (A and D) and in the nuclei of PCa cells (G and J) despite sparse nuclear staining in stromal cells (indicated by light blue arrow heads in figures D and J). ER β 1 staining was lost in some PCa foci (K). ER β 2 staining is highly cytoplasmic in both benign (basal and luminal epithelial cells, B and E) and PCa foci (H and L). Immunopositive signals of ER β 5 were observed in the cytoplasmic region of the epithelial cells in adjacent normal foci (C and F) as well as in PCa cells in Gleason score 6 cancer (I and M).

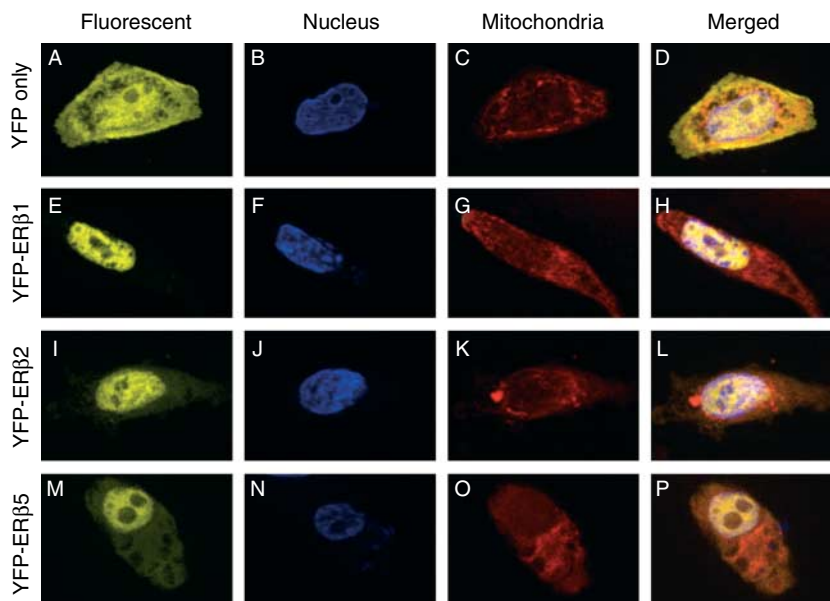


Figure 3 Subcellular localization of ER β isoforms in PC3 cells. Vectors carrying *ER β 1* (E–H), *ER β 2* (I–L), and *ER β 5* (M–P) were transiently transfected into PC3 cells, and expressed in the form of fusion protein with an N-terminal YFP tag. Cells transfected with YFP only serve as a control (A–D). Cells were counterstained with nucleus-specific (DAPI, B, F, J, and N) and mitochondria-specific fluorescent dyes (Mito-tracker red, C, G, K, and O). Subcellular localization of each isoform was determined by fluorescence microscopy. Extended focus was applied to average and combine three optical sections of each signal. Merged images of YFP, DAPI, and Mito-tracker red signals are shown in D, H, L, and P.

Characteristics of the patients whose prostates were used to construct the TMA

The majority of the patients ($n=133$) were Caucasian–Americans (92.4%; Table 1). Only 11 were non-Caucasian: African–American (3.5%), Hispanic (2.8%), or Asian (1.4%). Ages ranged from 46 to 77 years (mean=62). Approximately 44% of the patients had low-grade cancer (Gleason score <7), 40% had intermediate-grade PCa (Gleason score =7), and 16% had higher-grade cancer (Gleason score >7). The postoperative PSA levels of patients were measured for an average of 6.2 years. PF was detected in 37.5% of the patients ($n=54$). POM was found in 8.3% of the patients ($n=12$). The average time to PF and POM was 6.2 and 8.6 years respectively.

TMA analyses

To elucidate the clinical relevance of the expression of ER β 1, ER β 2, and ER β 5 in PCa, we used our newly validated antisera and ER β 1-specific antibody (GC17) in an IHC analysis of a set of TMAs comprising 144 specimens with a well-documented clinical history. Figure 4 shows typical results of IHC staining with ER β 1, 2, or 5 antibody/antisera. Typical TMA sections showing low to moderate staining of ER β isoforms are

shown in Fig. 4A, D, and G. In a few rare instances, both nuclear and cytoplasmic staining of ER β isoforms was observed in some of the TMA sections (Fig. 4B, E, and H). With higher magnification ($\times 630$), nuclei in PCa cells were differentially stained by the isoforms – antibody/antisera (Fig. 4C, F, and I). We noted different staining patterns in some specimens of similar cancer grade even with the same antibody/antiserum and IHC conditions. Variations were greater in the specimens positive for ER β 1 and ER β 5. The pattern for ER β 2 was less variable.

Distinct subcellular localization patterns of ER β isoforms in PCa specimens

ER β 1, 2, and 5 showed distinctly different expression patterns in this set of PCa TMAs. ER β 2 was found to be the dominant ER β isoform in PCa (Fig. 5). Over 80% of the samples ($n=127$) showed a positive ER β 2 signal. Most of the ER β 2 ($n=108$) was localized in the cytoplasm of the PCa cells, but $\sim 10\%$ resided in the nucleus. ER β 1 was the second most common isoform in this set of TMAs. Since 84% of the patients ($n=121$) had intermediate-grade PCa (Gleason 5–7) at prostatectomy, in agreement with previous studies, most of their prostate specimens still retained ER β 1 staining (Fig. 2; Leav *et al.* 2001). In contrast to the

Table 1 Characteristics of 144 patients

Variables	n (% total patients)
Age (years)	
46–50	4 (2.8)
50–59	49 (34.0)
60–69	81 (56.3)
70–77	10 (6.9)
Race	
Caucasian–American	133 (92.4)
African–American	5 (3.5)
Hispanics	4 (2.8)
Asian	2 (1.4)
Pathology stage	
T2	115 (79.9)
T3	29 (20.1)
Gleason score	
<7	63 (43.8)
=7	58 (40.2)
>7	23 (16.0)
Surgical margin	
Positive	58 (40.3)
Negative	86 (59.7)
PSA failure	
Yes	54 (37.5)
No	90 (62.5)
Metastasis	
Detected	12 (8.3)
Not detected	132 (91.7)

exclusive nuclear localization of ER β 1 in basal cells of benign glands, ER β 1 was found more often in both the nuclear and cytoplasmic compartments or in only the cytoplasmic compartment of PCa cells. ER β 5 is the least common isoform; only 36% of patients ($n=52$) showed ER β 5 positivity, which was predominantly expressed in the cytoplasm of the cancer cells (>90% of the cases, $n=49$).

Since we had previously shown that the various ER β isoforms formed heterodimers with ER β 1 in cell culture under different conditions of ligand stimulation (Leung *et al.* 2006), we calculated Spearman correlation analyses to determine the possibility of finding two of the three ER β s in the same cellular compartment in the PCa specimens (Table 2). Despite low-to-moderate correlations, a significant difference from zero was found for the correlation between ER β 1 and ER β 2 in the nucleus ($r=0.45$) and the cytoplasm ($r=0.39$; $P<0.01$), suggesting that ER β 1 and ER β 2 may be coexpressed in the nuclei or cytoplasm of PCa cells. ER β 5, which is predominantly cytoplasmic, therefore showed a better correlation only with cER β 1 ($r=0.37$) and cER β 2 ($r=0.26$; $P<0.01$), implying that some specimens may coexpress ER β 5 with at least one other ER β .

Statistical results of TMA studies

nER β 2 and cER β 5 Ascores were significantly related to PF and/or POM. Ascores were dichotomized as negative (≤ 3) and positive (> 3) based on statistical assessment and results of a previously published study (Shaaban *et al.* 2008). Descriptive statistics evaluating differences between dichotomized Ascores and clinicopathological features of PCa showed that age, preoperative PSA, and Gleason score were significantly different ($P<0.10$) for at least one isoform (nER β 2 and cER β 5; Table 3). Positive expression of nER β 2 was significantly associated with PF (adjusted odds ratio (adjOR)=3.8, $P=0.04$) and POM (adjOR=3.1, $P=0.02$), while cER β 5 positivity did not show a significant association with either PF (adjOR=0.8, $P=0.58$) or POM (adjOR=3.1, $P=0.09$; Table 3). Significantly poorer survival (time to POM), independent of Gleason score, age, and preoperative PSA, was associated with nER β 2 positivity (adjusted hazard ratio adjHR=4.6, $P=0.02$). These results suggest that nER β 2 is an independent prognostic marker for predicting time to POM. Time to PF was not significantly associated with positivity of nER β 2 or cER β 5 ($P>0.05$). Kaplan–Meier plots and the log-rank test statistics showed that time to PF and time to POM were significantly shorter for patients with nER β 2 positivity ($P<0.01$; Fig. 6A and B). cER β 5 positivity, on the other hand, was significantly

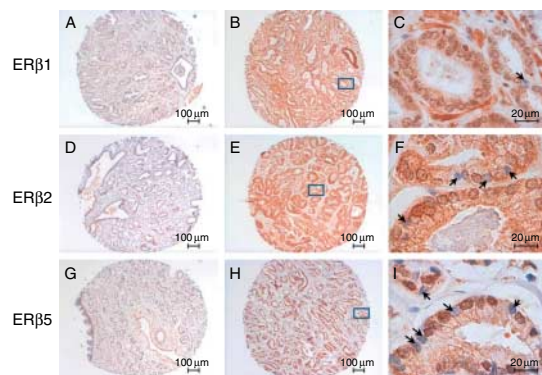


Figure 4 Typical IHC results of ER β immunostaining on TMAs. TMAs with 144 specimens were immunostained with ER β 1 (A–C), ER β 2 (D–E), and ER β 5 (G–I) antibodies/antisera. The Allred scoring system (Allred score = signal score + intensity score) was used to grade the immunostaining signals. Typical low Allred-scored sections (Allred = 0 + 1 for nuclear positivity; Allred = 1 + 2 to 1 + 3 for cytoplasmic positivity) are shown in A, D, and G. Higher Allred-scored sections (Allred = 3 + 5 for both nuclear and cytoplasmic positivity) with strong nuclear and cytoplasmic staining are shown in B, E, and H. C, F, and I represent a magnified view ($\times 630$) of a region (marked by a rectangle) in B, E, and H respectively. Negative nuclear staining in C, F, and I is marked by solid arrows.

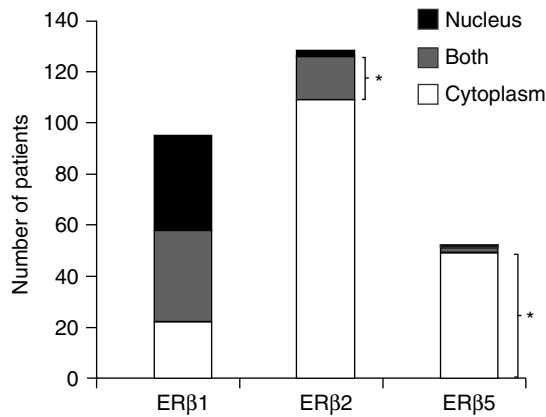


Figure 5 Distribution of ERβ isoforms in this prostate cancer TMA. The immunopositivity signal of each isoform was graded according to the Allred scoring system. Only an Allred score greater than cutpoint (i.e. Allred > 3) was considered positive. The number of patients showing positive immunostaining signals in different cellular compartments was determined and analyzed. The correlations between two ERβ isoform expression were determined based on Spearman correlation test. $P < 0.05$ was considered to be statistically significant. An asterisk represents a specific group of patients with a higher possibility of showing the worst clinical outcome.

associated only with a reduced survival time to POM ($P = 0.03$; Fig. 6C) but not time to PF ($P > 0.05$, data not shown). In a combined analysis, patients whose prostate specimen was positive for both nERβ2 and cERβ5 had the shortest POM-free survival ($P < 0.01$, degrees of freedom = 3), i.e. worst clinical outcome (Fig. 6D). Comparisons among pairs of groups revealed that the group with a double positive for nERβ2 and cERβ5 was significantly different from the group with a double negative ($P < 0.01$) and with the group with only nERβ2 positive ($P = 0.04$).

Migration and invasion of PCa cells expressing ERβ isoforms

On the basis of the results from the TMA study suggesting that both ERβ2 and ERβ5 could be the markers for predicting time to POM, we further investigated the role of these two isoforms in PCa

metastasis. We stably infected *LacZ*, *ERβ1*, *ERβ2*, and *ERβ5* in PC3 cells with lentivirus, and measured cell mobility and invasion ability of the cells expressing different isoforms with wound-healing and invasion assays respectively. Stable ectopic expression of each ERβ isoform in PC3 cells was confirmed with established real-time PCR assays (Leung *et al.* 2006). A greater than 200-fold increase in ERβ transcript was detected in each stable-transfected cell line. In wound-healing assays, PC3-ERβ5 cells migrated significantly faster than the others after 24 h of wound introduction (Fig. 7A). PC3-ERβ2 cells migrated as fast as the PC3-LacZ control cells, but PC3-ERβ1 cells migrated significantly more slowly than control cells. In invasion assays, the efficiency of invasion across the matrix gel membrane of PC3-ERβ2 and PC3-ERβ5 cells was 64 and 42% respectively greater than that of PC3-LacZ cells within 24 h (Fig. 7B). Interestingly, ectopic expression of ERβ1 in PC3 cells significantly reduced PCa cell invasiveness by 34% as compared with PC3-LacZ.

Discussion

This is the first study comparing the protein expression of ERβ1 with that of its isoforms ERβ2 and ERβ5 in specimens from a cohort of patients with PCa ($n = 144$) with long clinical follow-up. The immunohistochemical expression of the three ERβs was analyzed in a set of TMAs derived from the cohort using an ERβ1-specific antibody and two newly characterized antisera for ERβ2 and ERβ5. nERβ2 was found to be an independent predictor for PF (adjOR = 3.8, $P = 0.04$), POM (adjOR = 3.1, $P = 0.02$), and POM-free survival (adjHR = 4.6, $P = 0.02$), whereas cERβ5 was a predictor for POM-free survival in Kaplan–Meier analysis. More importantly, patients positive for both ERβ isoforms (nERβ2+ and cERβ5+) were found to have the shortest POM-free survival ($P < 0.01$). To provide mechanistic insights into the clinical relevance and prognostic significance of both isoforms, we ectopically expressed ERβ isoforms in PC3 cells and

Table 2 Spearman correlation $S(r)$ between expression of estrogen receptor β1 (ERβ1), ERβ2, and ERβ5 isoform

	n^a	Nucleus		Cytoplasm	
		r	P value	r	P value
ERβ1 versus ERβ2	134	0.45	<0.01	0.39	<0.01
ERβ1 versus ERβ5	134	0.10	0.23	0.38	<0.01
ERβ2 versus ERβ5	134	0.06	0.48	0.24	<0.01

P values testing $r = 0$.

^aPatient numbers are <144 due to missing data.

Table 3 Descriptive statistics and results of regression analyses

	Nuclear ER β 2 ($n=136^a$)			Cytoplasmic ER β 5 ($n=137^a$)		
	Positive ^b (%)	Negative ^b (%)	P value	Positive ^b (%)	Negative ^b (%)	P value
<i>Clinicopathological features of PCa</i>						
Number of patients (n)	21	115		56	81	
Age (years)						
Mean \pm s.d.	59.5 \pm 5.9	62.3 \pm 6.1	0.06	62.3 \pm 6.1	61.7 \pm 6.3	0.58
Preoperative PSA (ng/ml)						
Median (min, max)	5.0 (3.0, 20.1)	6.8 (0.05, 32.3)	0.03	6.6 (0.3, 32.3)	6.2 (0.05, 23.2)	0.71
Gleason score						
<7	4 (19.1)	53 (46.1)		22 (39.3)	36 (44.4)	
=7	13 (61.9)	44 (38.3)	0.06	22 (39.3)	35 (43.2)	0.36
>7	4 (19.1)	18 (15.7)	(df=2)	12 (21.4)	10 (12.4)	(df=2)
Pathology stage ^c						
T2	19 (90.5)	89 (77.4)		43 (76.8)	66 (81.5)	
T3	2 (9.5)	26 (22.6)	0.14	13 (23.2)	15 (18.5)	0.50
Surgical margins						
Positive	7 (33.3)	50 (43.5)		25 (44.6)	32 (39.5)	
Negative	14 (66.7)	65 (56.5)	0.47	31 (55.4)	49 (60.5)	0.60
PSA failure						
Yes	14 (66.7)	39 (33.9)		22 (39.3)	31 (38.3)	
No	7 (33.3)	76 (66.1)	<0.01	34 (60.7)	50 (61.7)	0.90
Post-operative metastasis						
Yes	5 (23.8)	7 (6.1)		8 (14.3)	4 (4.9)	
No	16 (76.2)	108 (93.9)	<0.01	48 (85.7)	77 (95.1)	0.06
Outcome	adjOR	adjHR	P value	adjOR	adjHR	P value
<i>Adjusted odds ratio (adjOR^d) and hazard ratio (adjHR^d)</i>						
PSA failure	3.8		0.04	0.8		0.58
Time to PSA failure		1.8	0.10		0.8	0.47
Post-operative metastasis	3.1		0.02	3.1		0.09
Time to post-operative metastasis		4.6	0.02		3.1	0.07

P values test i) differences in mean age and median preoperative PSA between positive and negative ER β isoforms; ii) differences in Allred positivity rates among categories of Gleason score, pathology stage, and surgical margins.

^aTable entries for count data indicate number of patients (%column total). Column totals are <144 due to missing data.

^bCutpoint for positive: Allred score >3; cutpoint for negative: Allred score \leq 3.

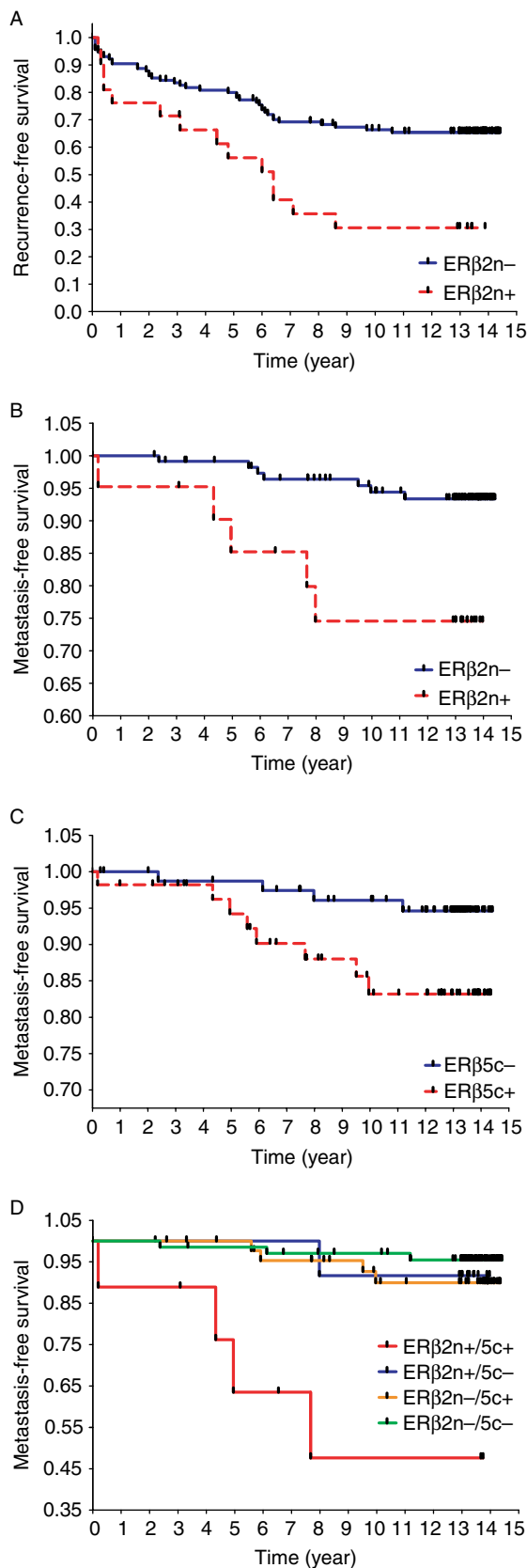
^cNo stages 1 and 4 were detected.

^dAdjOR measures the odds of a positive isoform from logistic regression, adjusted for age, Gleason score, and preoperative PSA; AdjHR measures the hazard (or risk) of a positive isoform from a Cox proportional hazards model with the same adjustments.

found that stable expression of these isoforms in this cell line increased the efficiency of cell migration (for PC3-ER β 5 cells) and cell invasiveness (for both PC3-ER β 2 and PC3-ER β 5 cells) but did not alter cell proliferation (unpublished data). Taken together, these findings established that ER β 2 and ER β 5 are strongly associated with PCa metastasis.

Estrogen has been shown to be involved in normal and malignant functions of the prostate (Ho et al. 2006). Four decades ago, the oral estrogen diethylstilbestrol was the treatment of choice for PCa (Huggins & Hodges 2002). It was ultimately abandoned because of its serious cardiovascular and thromboembolic toxicity (Hanash et al. 1970, Eisen

et al. 1975) and the emergence of gonadotropin agonists/antagonists (Schally et al. 1983). However, other forms of estrogen, including various ER selective modulators, recently have emerged as effective and economical therapies, largely because of clinical data showing minimal hepatic toxicity of these agents if they are administered parentally via an intramuscular or transdermal route (Ockrim et al. 2006). These therapies also have significant efficacy in protecting against osteoporotic fracture, hot flashes, asthenia, and cognitive dysfunction in patients with PCa (Ockrim et al. 2006). With this renewed interest in using estrogens as single or combination therapy for PCa, the need to understand how ERs affect PCa is pressing.



Results from this study serve as the first exploration to open a new line of investigation.

The action of estrogen in human normal and malignant prostatic tissue is believed to be mediated partly by ERβ because it is the predominant ER subtype in the prostate epithelium (Kuiper *et al.* 1996, Leav *et al.* 2001). However, the role of ERβ in prostate and breast cancers is still debatable, even after 10 years of extensive research (Ho *et al.* 2006). Data from different research groups conflict (Speirs *et al.* 2004, Skliris *et al.* 2006), in part because of insufficient understanding of ERβ isoforms that were discovered relatively recently (Leung *et al.* 2006) and the lack of antibodies to distinguish them *in vitro* and in clinical specimens. Unlike breast cancer research, in which the discovery of these molecules has stimulated new research directions (Speirs & Walker 2007, Powell & Xu 2008), similar developments in PCa research have been limited. The use of N-terminal-specific ERβ antibodies in IHC experiments is always problematic because they recognize all ERβ isoforms, and mRNA expression data clearly showed that each ERβ isoform has a unique, tissue- and cell type-specific expression pattern (Leung *et al.* 2006). This study is the first to provide data at the protein level illustrating this uniqueness in PCa. Our IHC staining revealed unique tissue-/cell type- and cellular compartment-specific distribution for each isoform in the normal and malignant prostate.

The functions of each isoform in the normal prostate epithelium are unclear. Thus, in this study, ERβ1 positivity was not associated with PCa progression, but the PC3 cell model suggested that ERβ1 inhibits both PCa cell migration and invasion, which is consistent with the current belief that ERβ is antiproliferative, tumor suppressive (Morani *et al.* 2008) and impedes epithelial–mesenchymal transition (Mak *et al.* 2010). However, others have suggested that ERβ is involved in an aggressive PCa phenotype (Horvath *et al.* 2001,

Figure 6 Evaluation of ERβ isoforms as a predictor for time to PSA failure and time to metastasis by the Kaplan–Meier (KM) plot with the log-rank test. The Allred cutpoint (>3 or ≤ 3) for each ERβ isoform was used to determine ‘positive’ and ‘negative’ expression. (A) KM plot of nuclear ERβ2 versus recurrence/PSA-free survival ($P < 0.01$). (B) KM plot of nuclear ERβ2 versus metastasis-free survival ($P < 0.01$). (C) KM plot of cytoplasmic ERβ5 in metastasis-free survival ($P = 0.03$). (D) KM plot of nuclear ERβ2 and cytoplasmic ERβ5 versus metastasis-free survival ($P < 0.01$). Patients were further stratified into four groups according to status of ERβ isoform expression: i) both nuclear ERβ2 and cytoplasmic ERβ5 positive ($n = 9$); ii) nuclear ERβ2 positive and cytoplasmic ERβ5 negative ($n = 12$); iii) nuclear ERβ2 negative and cytoplasmic ERβ5 positive ($n = 46$); and iv) both nuclear ERβ2 and cytoplasmic ERβ5 negative ($n = 68$). $P < 0.05$ was considered to be statistically significant.

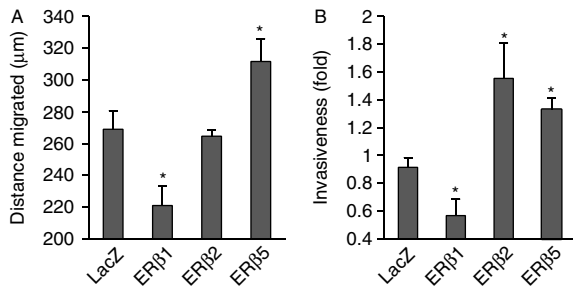


Figure 7 Tumor metastasis of PC3 expressing ER β isoforms. (A) Results of wound-healing assay. Migration distance of PC3 expressing LacZ (as a control), ER β 1, ER β 2, and ER β 5 was recorded and calculated after 24 h of wound introduction. Experiments were carried out in triplicate and were repeated with three independent sets. Student's *t*-test was used to compare the mean distance migrated of each ER β isoform-expressing cell line versus LacZ control cells (average \pm S.E.M., **P* < 0.05). (B) Results of Matrigel-based invasion assay. Fixed number (5×10^4) of cells was set up in a 24-well plate according to the manufacturer's recommendation. The cells that crossed the matrigel membrane were stained and counted under a microscope. Cell numbers were normalized by the MTS method. The fold invasiveness was calculated relative to the number of cells invaded in LacZ control. Experiments were carried out in triplicate and were repeated with three independent sets. Student's *t*-test was used to compare the fold invasiveness of each cell line versus LacZ control cells (average \pm S.E.M., **P* < 0.05).

Nanni *et al.* 2009). We speculate that this kind of discrepancy will be resolved in the future by including ER β isoforms during data analysis. In this TMA study, ER β 2, the most commonly expressed isoform (> 80%) in PCa cells, is primarily cytoplasmic, and its nuclear positivity correlates with PF, POM, and a shorter time to POM. These results are in agreement with a study with a smaller cohort analyzed with different clinical outcomes (Fujimura *et al.* 2001). However, this study was the first to investigate ER β 5 and its prognostic value in PCa. Both ER β 2 and ER β 5 elevated PCa cell invasion, but only the cells expressing ER β 5 migrated more rapidly. This suggests that ER β 2 and ER β 5 could regulate different tumor metastasis pathways in PCa cells, perhaps explaining why co-existence of ER β 2 and ER β 5 in PCa tissue could predict the worst clinical outcome, probably due to synergistic actions of ER β 2 and ER β 5 in PCa progression. Through the use of isoform-specific antibodies, we have gained new insights into the prognostic values of ER β isoforms that will undoubtedly continue to increase our understanding of ER β s in the development and progression of PCa. The utility of ER β 2 and ER β 5 as PCa prognostic markers is obviously worthy of further investigation.

Both isoforms have been found in various tissues (Pedersen *et al.* 2001, Scobie *et al.* 2002, Poola 2003,

Cammarata *et al.* 2005, Wong *et al.* 2005), including the prostate (Leung *et al.* 2006). The functions of these isoforms are not yet clear, but they are known to be involved in ER α (Peng *et al.* 2003, Poola *et al.* 2005) and ER β 1 signaling (Leung *et al.* 2006). At the molecular level, either ER β 2 or ER β 5 by itself does not transactivate ERE-driven promoter but when heterodimerized with ER β 1 can alter its transactivation activity (Leung *et al.* 2006). The roles of ER β 2 and ER β 5 in cancer were not studied until recently. Their nuclear expression in breast cancer tissue was significantly correlated with better overall survival (Shaaban *et al.* 2008), a finding that differs from our data showing a significant correlation between ER β 2/ER β 5 positivity in PCa cells and poor POM-free survival. This discrepancy suggests a fundamental difference in the role played by estrogens/estrogen signaling in the pathogenesis of PCa versus that of breast cancer, which has introduced an intriguing question regarding the clinical management of these cancers.

We recognize that our cohort is small (144 patients) compared with that of the breast cancer study (Shaaban *et al.* 2008) and thus includes relatively few patients with PF (*n* = 54; ≥ 2 ng/ml) and POM (*n* = 12). However, the possibility of using ER β 2 and ER β 5 for PCa prognosis shows great promise, partly because of the long lead-time before postoperative PF (6.2 years) and time to POM (8.6 years). Therefore, the confirmation of ER β 2, in conjunction ER β 5 or other established markers, as an independent prognostic marker in future cohort studies, could help to identify candidates for clinical trials of new interventions designed to curb PCa progression based on information gained at the time of radical prostatectomy from IHC analysis for ER β s. Moreover, more active surveillance and/or adjuvant therapies, such as external beam radiotherapy and ADTs (Michaelson *et al.* 2008, Wirth *et al.* 2008), may be indicated for the subset of patients at higher risk for metastases because of their positivity for ER β 2 and ER β 5.

Mechanistic studies aimed at elucidating the biological roles of ER β s require relevant cell model systems. Cammarata *et al.* (2005) were the first to report different subcellular localizations of ER β isoforms in breast cancer cells. In a PCa cell line (PC3), ER β 1 resides mostly in the nucleus. Although very few ER β 1 molecules were detected in cytoplasm, they were not localized in the mitochondria in this PCa cell line, a finding different from other published cell data (Chen *et al.* 2004). Both ER β 2 and ER β 5 were localized in both cytoplasmic and nuclear compartments in PC3 cells. It is not clear whether such unique patterns of

subcellular localizations of ER β isoforms in PCa cells are related to their metastasis-promoting functions or simply to differential protein trafficking and/or processing. Those are the questions worthy of further investigation. As a caveat, it is almost certain that there are no perfect cell model systems that could fully recapitulate the *in vivo* situation, whereby epithelial cells are surrounded by different types of stromal cells in a three-dimensional architecture. Thus, interpretations of data from cell model systems would best be supported by parallel correlative studies of human tissues.

In conclusion, in a group of PCa patients with relatively long follow-up, our data demonstrate that expression of ER β 2 in the nucleus of PCa cells is an independent prognostic marker of PF, POM, and time to POM, and cER β 5 positivity predicts shorter POM-free survival. Additionally, the expression of both nER β 2 and cER β 5 identified a group of patients with the worst clinical outcome. Finally, forced expression of ER β 2 or ER β 5 in PC3 cells uncovered that these isoforms have metastasis-promoting action, supporting our IHC findings. Given the long duration of time to PF/POM, prognostic markers such as these should help in stratifying patients in clinical trials and in devising more effective customized therapies for advanced PCa.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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