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Prostate cancer expression profiles of cytoplasmic ERβ1 and nuclear ERβ2 are associated with poor outcomes following radical prostatectomy

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Abstract

Purpose—Existing data regarding the expression of estrogen receptors (ER) and prostate cancer outcomes have been limited. We evaluated the relationship of expression profiles of ERβ subtypes and the estrogen receptor GPR30 with patient factors at diagnosis and outcomes following radical prostatectomy.

Materials and Methods—Tissue microarrays constructed from 566 men with long-term clinical follow-up were analyzed with immunohistochemistry targeting ERβ1, ERβ2, ERβ5 and GPR30. An experienced pathologist scored receptor distributions and staining intensities. Tumor staining characteristics were evaluated for associations with patient characteristics, recurrence free survival, and prostate cancer-specific mortality (PCSM) following radical prostatectomy. Results: Prostate cancer cells had unique receptor subtype staining patterns with ERβ1 demonstrating predominantly nuclear localization, while ERβ2, ERβ5 and GPR30 were predominantly cytoplasmic. After controlling for patient factors, intense cytoplasmic ERβ1 staining was independently associated with time to recurrence (HR 1.7, 95% CI 1.1-2.6, p=0.01) and PCSM (HR 6.6, 95% CI 1.8-24.9, p=0.01). Similarly, intense nuclear ERβ2 staining was independently associated with PCSM (HR 3.9, 95% CI 1.1-13.4, p=0.03). Patients with cytoplasmic ERβ1 and

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nuclear ERβ2 co-staining had significantly worse 15-year PCSM vs. patients expressing only cytoplasmic ERβ1, only nuclear ERβ2, or neither (16.4% vs. 4.3% vs. 0.0% vs 2.0 %, respectively p=0.001).

Conclusions—Increased cytoplasmic ERβ1 and nuclear ERβ2 expression are associated with worse cancer-specific outcomes following radical prostatectomy. These findings suggest that tumor ERβ1 and ERβ2 staining patterns provide prognostic information for radical prostatectomy patients.

Keywords

Prostate Cancer; Estrogen Receptor; Prostatectomy

Introduction

Prostate cancer (PCa) is the second leading cause of male cancer-specific mortality in the United States, with approximately 30,000 deaths annually.[1] Although many men die from PCa, the majority of men diagnosed in the PSA era have low-risk disease from which many never develop symptoms of their disease irrespective of treatment. This wide spectrum of disease behavior underscores the need for biomarkers of PCa-specific outcomes to accurately predict PCa prognosis and tailor treatment to each patient's disease.

One potential biomarker is estrogen activity, whose role in prostate carcinogenesis and treatment has been evaluated extensively.[2] The main effectors of estrogen signaling are the estrogen receptors (ER)[3], of which ERβ is the dominant receptor in the prostate epithelium.[4] ERβ has been hypothesized to play an anti-proliferative role within the prostate based on the inhibition of cellular proliferation in PCa cells,[5] the development of hyperplasia and dysplasia in ERβ knockout mice,[6,7] the reduction in ERβ expression in high-grade intraprostatic neoplasia vs. benign glands,[8,9] and the marked decrease in ERβ expression in various high vs. low-grade tumors.[8,10] However, ERβ expression has been observed in the majority of nodal and boney metastases[8,9] and has been associated with worse overall survival among hormone naïve patients initiating androgen deprivation therapy. [11]

Recent identification of ERβ isoforms provides insights into the complex biological outcomes. In addition to the wild-type (WT) ERβ (ERβ1), humans have four splice variants, ERβ2-5.[12] ERβ1 is the only fully functional receptor, while ERβ2, β4 and β5 heterodimerize with ERβ1 regulating its transactivation.[13] The function of ERβ isoforms as mediators of estrogen signaling suggests that ERβ isoform expression patterns could influence the biology of malignant cells and in turn, have a prognostic role[14].

We evaluated the relationship between ERβ isoform expression patterns and oncologic outcomes in men with localized PCa treated with radical prostatectomy (RP). Additionally, based on recent data suggesting that stimulation of the estrogen-binding G-protein-coupled receptor-30 (GPR30) inhibits the growth of PCa both *in vitro* and *in vivo* in murine xenografts[15] we also evaluated the association between GPR30 expression and post-RP outcomes.

Methods

Study Population

We performed a retrospective review of men who underwent RP for histologically confirmed clinically localized PCa and were previously enrolled in population-based studies of PCa in King County, WA[16,17] The first study ascertained cases under age 65 years who were diagnosed between 1993 and 1996, and for the second study, men were under age 75 and were diagnosed between 2002 and 2005. These studies included N=831 patients who were identified from the Seattle-Puget Sound SEER cancer registry and underwent structured in-person interviews conducted by trained staff to collect demographics and past medical history data as previously described.[16,17] Formalin-fixed paraffin embedded (FFPE) blocks of tumor tissue were available in N=566 interviewed patients and made up the present study population. Follow-up surveys were completed by patients between 2004 and 2005 and 2010 and 2011. Clinical data including Gleason score, pathologic stage, PSA at diagnosis, and primary therapy were obtained from the Seattle-Puget Sound SEER cancer registry. Vital status as of December 2013 and underlying cause of death were determined through linkage with the SEER registry and review of death certificates. Recurrence was defined as PSA ≥ 0.2 ng/mL; positive bone scan, computed tomography, and/or magnetic resonance imaging; positive lymph node or prostate bed biopsy; receipt of secondary or salvage therapies; physician statement of PCa recurrence, spread and/or PCa-specific mortality (PCSM) following RP. The study received approval from the Fred Hutchinson Cancer Research Center Institutional Review Board and all patients provided informed consent.

Construction of Tissue Microarrays and Immunohistochemistry

Hematoxylin and eosin slides were made from FFPE blocks of tumor tissue obtained at the time of RP. An experienced genitourinary pathologist (X.Z.) reviewed the slides and marked regions containing ≥75% tumor. Duplicate 1.0 mm diameter cores were taken from the dominant tumor focus in the corresponding region of the block and arrayed into a new recipient paraffin block. Five-micron tissue microarray (TMA) sections were then cut, deparaffinized, and rehydrated in dH_2O. TMA immunohistochemistry was performed for the detection of ERβ1, ERβ2, ERβ5, and GPR30.[14] The dilution ratio for the primary antiserum was 1:100 for ERβ1 and ERβ5, and 1:500 for ERβ2.[14,18] Anti-GPR30 antibody (ab12563) was purchased from Abcam (Cambridge, MA) and the dilution ratio was 1:100.

The immunostaining of each receptor within the cancerous glands was scored by an experienced pathologist (X.Z.) blinded to clinical parameters. Cytoplasmic and nuclear staining were evaluated separately. Tissue cores with unsatisfactory staining, uncertain histology, or that were missing/damaged were excluded from the analysis. Intra-observer concordance was evaluated by rescoring a randomly selected 2% sample by the study pathologist. Intra-patient concordance was evaluated on each of the two core samples from the same 2% sample. As previously described,[19] immunostaining was assessed using a score created by multiplying staining intensity (0 for no staining, 1 for light staining, and 2 for strong staining) by the corresponding percentage of cells staining positive at each intensity. The mean score was used for cases with data from duplicate cores. Based on the distribution

of staining intensities, weak staining was defined as a staining score of >0 to <1 and intense staining as a staining score ≥1.

Statistical Analysis

Associations between cytoplasmic and nuclear ERβ1, ERβ2, ERβ5, and GPR30 staining and clinicopathologic data were analyzed with the Chi^2 test. Associations between receptor staining profiles and PCa outcomes were evaluated with the Kaplan-Meier method and the log-rank test. Receptors found to be significantly associated with time to recurrence or PCSM were then evaluated in multivariable Cox proportional hazards models adjusted for age, diagnostic PSA, Gleason score, and pathologic stage. Significance was set at <0.05, two-tailed test. All analyses were performed with Stata SE/12 (College Station, TX).

Results

Descriptive statistics for the study population are outlined in Table 1. The median diagnostic PSA was 6.0 (± 10.2) ng/mL, 290 (51.2%) men had ≥ Gleason grade 7, and 390 (68.7%) had organ-confined (pT2) disease (Table 1). Among survivors, the median follow-up was 10.5 years (range 0.7 – 20.3 years).

Unique expression was observed for each receptor (Figures 1 and 2). Tumors expressed ERβ1 in 95.7%, ERβ2 in 97.5%, GPR30 in 97.5%, and ERβ5 in 22.9% of interpretable patients. ERβ1 staining was nuclear only in 316 (62.1%), cytoplasmic only in 43 (8.5%), and in both the nucleus and cytoplasm in 128 (25.1%) patients. ERβ2 staining was nuclear only in 5 (1.0%), cytoplasmic only in 376 (72.0%), and in both in 129 (23.7%) patients. Conversely, ERβ5 and GPR30 demonstrated minimal heterogeneity with nearly exclusive cytoplasmic staining. Intra-observer and intra-patient concordance were ≥85% for all receptors. Associations between receptor staining and patient clinicopathologic data are presented in Table 2. The staining distribution of cytoplasmic ERβ1 (cERβ1) was significantly different (p=0.04) between patients with localized disease (none: 67.5%, weak: 11.9%, intense: 20.5%) vs. regional disease (none: 64.0%, weak: 6.7%, intense: 29.2%). No associations were observed between clinical data and ERβ2, ERβ5, or GPR30 staining.

Clinical Outcomes

PCa recurrence information was available on 460 patients (81.3%, 18.7% of patients did not return follow-up questionnaires) in which there were 119 (25.9%) recurrences, correlating to a 5-year and 10-year recurrence free survival (RFS) probability of 85.0% and 72.6% following RP. On Kaplan-Meier analysis (Figure 3), cERβ1 staining intensity was significantly associated with shorter time to recurrence (p=0.004) with 5-year recurrence free survival (RFS) probabilities of 73.2% for intense vs. 91.1% for weak vs. 88.3% for no staining. nERβ2 staining intensity was not significantly associated with time to recurrence (p=0.11), however, when censored at 5-years intense nERβ2 staining intensity was associated with significantly worse 5-year RFS compared to weak or no staining (69.1 % vs. 81.4% vs. 88.1%, p=0.02). On multivariable analysis, intense cERβ1 staining was independently associated with time to recurrence (HR 1.7, 95% CI 1.1 – 2.6), p=0.01), when adjusting for patient age, Gleason score, pathologic stage and diagnostic PSA (Table 3).

PCa-specific death was observed in 13 (2.3%) men, resulting in a 3.2% estimated 15-year PCSM. Estimated 15-year PCSM probabilities were significantly different across cERβ1 staining strata at 9.7% vs. 2.1% vs. 1.4% (p=0.003) for intense vs. weak vs. no staining, respectively (Figure 3). Similarly,15-year PCSM probabilities were significantly different across nERβ2 staining strata at 11.3% for intense vs. 1.9% for weak vs. 2.7% for no staining, respectively (p=0.02). On unique multivariable analyses controlling for patient age, Gleason score, pathologic stage and diagnostic PSA, intense cERβ1 (HR 6.6, 95% CI 1.8 – 24.9, p=0.01) and intense nERβ2 (HR 3.9, 95% CI 1.1 – 13.4, p=0.03) staining were both associated with an increased risk of PCSM. We then evaluated the effect of cERβ1 and nERβ2 co-expression using the Kaplan-Meier method, demonstrating estimated 15-year PCSM of 16.4% vs. 4.3% vs. 0% vs. 2.0 % (p=0.001) for patients who expressed cERβ1 and nERβ2 vs. only cERβ1 vs. only nERβ2 vs. neither (Figure 4). ERβ5 and GPR30 staining distributions were not associated with RFS or PCSM.

Discussion

We evaluated the relationship between ER expression patterns and PCa outcomes in a population based cohort (n=566) of men with clinically localized disease undergoing RP with long-term follow-up. Intense expression of cERβ1 within tumors was independently associated with worse RFS (HR 1.7, 95% CI 1.1–2.6) and PCSM (HR 6.6, 95% CI 1.8-24.9) with significantly worse 5 year RFS (73.2% vs. 91.1% vs. 88.3% for intense vs. weak vs. none, respectively, p=0.004) and 15 year PCSM (9.7% vs. 2.1% and 1.4% for intense vs. weak vs. none, respectively, p=0.003). Similarly, intense staining of nERβ2 (HR 3.9, 95% CI 1.1-13.4) was independently associated with increased risk of PCSM with significantly worse 15 year PCSM (11.3% vs. 1.9% vs. 2.7% for intense vs. weak vs. no staining, respectively, p=0.02). Further, co-expression of cERβ1 and nERβ2 was associated with a significantly worse 15-year PCSM compared to patients expressing cERβ1 alone, nERβ2 alone, or neither. These data suggest that cERβ1 and nERβ2 may be useful prognostic biomarkers to identify men undergoing RP who are higher risk for adverse outcomes.

These findings contribute to the literature evaluating the role of ERβ in prostate carcinogenesis and prognosis. In particular, this study is the first to demonstrate a potential link between cERβ1 expression and adverse post-RP outcomes. These observations were not expected based on the hypothesized anti-proliferative effects of ERβ1[5] and a study by Leung et al, in which no associations between cERβ1 expression and PCa outcomes were observed using the same ERβ1 antibody to stain the tumors of 144 men undergoing RP.[14] However, ERβ1 staining distributions were highly similar in both studies suggesting that the interpretation of ERβ1 staining was accurate and does not account for the differences observed between cERβ1 and clinical outcomes. There were, however, important differences between our study and the study by Leung et al. which may account for discrepancies in the relationship between ERβ1 and post-RP outcomes. First, we controlled for pathologic stage, which is known to impact recurrence and survival[20,21] and was associated with cERβ1 staining intensity in our cohort. Second, we examined RFS and PCSM compared to PSA recurrence and post-RP metastases. Third, with longer follow-up and nearly quadruple the number of patients, our study had improved statistical power to detect relationships between ERβ subtypes and PCa-specific outcomes. Associations between cERβ1 staining and worse

post-RP outcomes are consistent with data demonstrating expression of ERβ1(or WT ERβ) in the majority of nodal and boney metastases[8,9] and PCa tumor expression of WT ERβ being associated with increased risk of recurrence following RP[22] and worse overall survival in hormone naïve patients with metastatic disease[11].

Our observations linking nERβ2 to poor PCa prognosis are consistent with previous studies. Specifically, the nearly 4-fold increased risk of PCSM among men with intense nERβ2 expression in this study is similar to the findings of Fujimura et. al. in which they identified elevated ERβ2 expression as a risk factor for PCSM in a cohort of 50 men.[23] Additionally, while nERβ2 staining was not significantly associated with overall RFS (p=0.11) in our cohort, intense nERβ2 staining was significantly associated with 5-year RFS (69.1 % vs. 81.4% vs. 88.1% for intense vs. light vs. no staining) on Kaplan-Meir analysis, similar to the study by Leung et al in which high nERβ2 expression was independently associated with increased risk of biochemical recurrence and post-operative metastases.[14] These clinical data are further supported by *in vitro* observations demonstrating increased invasiveness of PC3 cells expressing ERβ2.[14] Additionally, ERβ2 expression has been associated with increased cellular proliferation and the expression of proliferation associated genes both *in vitro* and in mouse engrafts with up-regulation of mediators of boney metastases.[24] Thus, biologic evidence supports increasingly aggressive cellular behavior with increasing ERβ2 expression in line with the observed poor outcomes following RP in patients with greater nERβ2 expression.

The impact of interactions between different ERβ isoforms on the biology of PCa is currently under investigation. However, evidence suggests that ERβ2 has no innate activity of its own and does not homodimerize, instead forming heterodimers with ERβ1 resulting in modulation of its activity.[12,13] Further, ERβ2 acts as a transcriptional repressor of ERβ1 (thus inhibiting its usual anti-proliferative effects)[25] suggesting that ERβ2 may therefore function as a dominant-negative regulator of ERβ1 via heterodimerization. As a result, one possible hypothesis to explain the observed association between intense cERβ1 staining and adverse post-RP outcomes is an interaction between ERβ1 and ERβ2 in the cytoplasm. As 99% of patients with ERβ2 staining had cERβ2, nearly all patients with cERβ1 were also cERβ2. Consequently, intense cERβ1 staining could identify those patients with the greatest degree of dominant negative heterodimerzation between cERβ1 and cERβ2, possibly preventing translocation of ERβ1 into the nucleus and thereby preventing the expected antiproliferative effects of ERβ1. Similar interactions between ERβ1 and ERβ2 could also account for the association observed between intense nERβ2 staining and increased PCSM, with nERβ2 forming dominant negative heterodimers with ERβ1 in the nucleus of tumor cells. As the current study cannot address this hypothesis, further studies are needed to evaluate the impact of ERβ1 and ERβ2 co-localization/interaction on ERβ signaling and subsequent downstream effects on PCa biology and patient outcomes. If such interactions were confirmed to be biologically important, selective ERβ isoform agonists/antagonists could potentially serve as new targeted agents in the management of PCa.

Limitations of this study include the potential for unmeasured confounding, however, our dataset includes clinical, pathological and epidemiological factors previously associated with PCa outcomes. Additionally, despite 566 patients in our cohort with relatively long median

follow-up (>10 years) among survivors, only 3.2% experience PCSM, limiting the statistical power. Even with these limitations, our results suggest that patients whose tumors express increased cERβ1 and nERβ2 are at particularly high-risk and may warrant closer surveillance following RP.

Conclusion

Men whose tumors highly express cERβ1 and/or nERβ2 may have increased risk of adverse PCa-specific outcomes following RP. If confirmed, these findings suggest that evaluation of ERβ1 and ERβ2 expression at the time of RP could provide important prognostic information and inform post-RP surveillance strategies.

Acknowledgments

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Abbreviations

ER	Estrogen Receptor
FFPE	Formalin Fixed Paraffin Embedded
PCa	Prostate Cancer
PCSM	Prostate Cancer Specific Mortality
RFS	Recurrence Free Survival
RP	Radical Prostatectomy
TMA	Tissue Microarray
WT	Wild Type

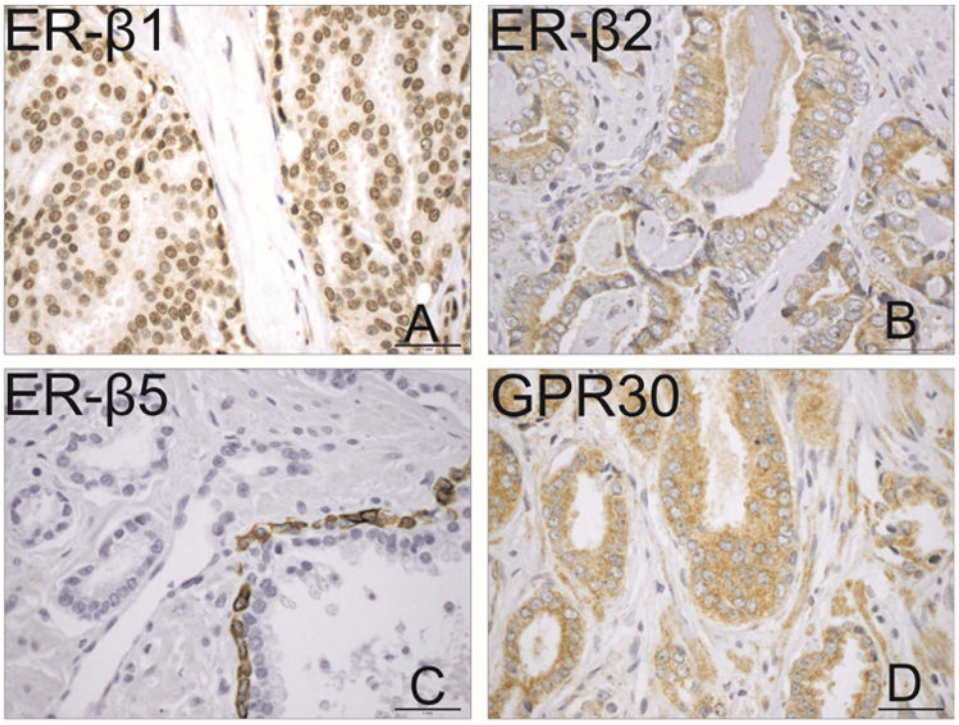

Figure 1.
Representative immunohistochemical staining of ERβ1 (A), ERβ2 (B), ERβ5 (C) and GPR30 (D) at 100× magnification.

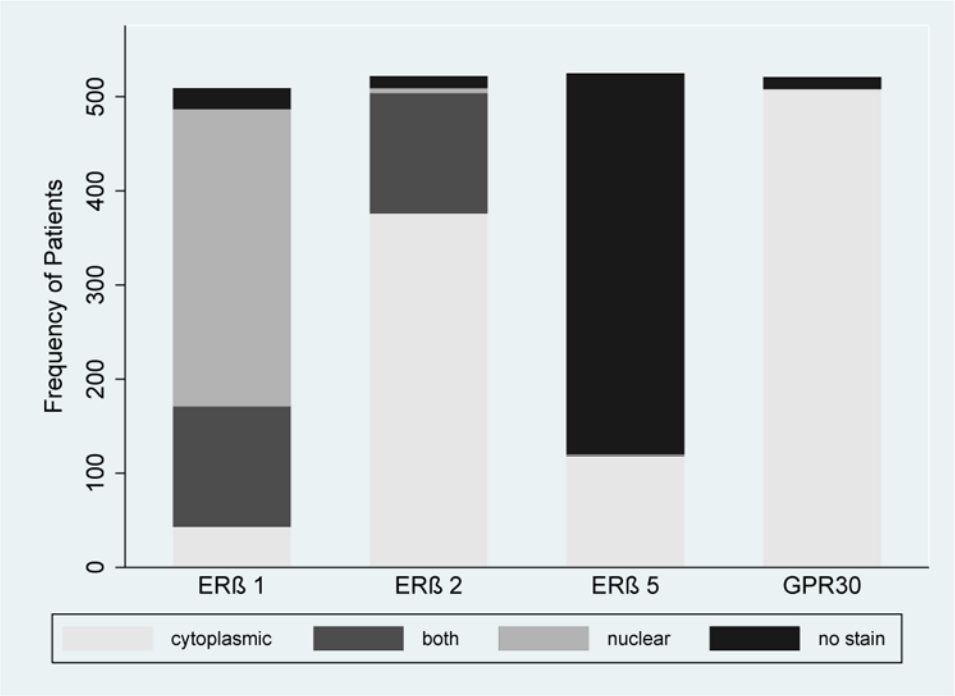

Figure 2.
Distribution of estrogen receptor staining (ERβ1, ERβ2, ERβ5 and GPR30) by location (any vs. no staining).

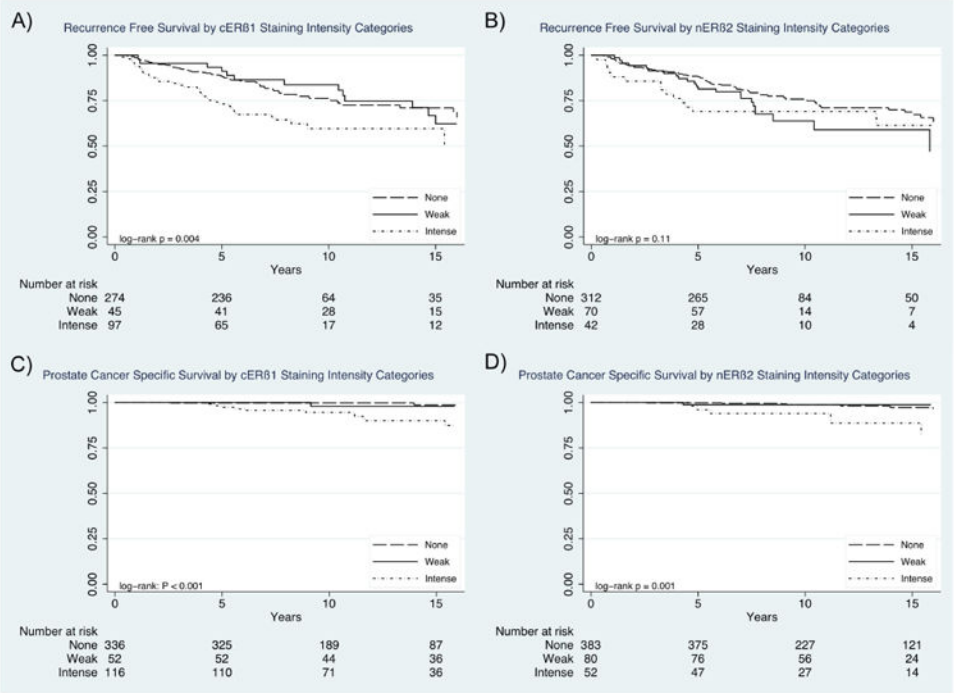

Figure 3.

Prostate cancer recurrence free probability (A, B) and prostate cancer-specific survival probability (C, D) stratified by cERβ1 and nERβ2 staining intensity.

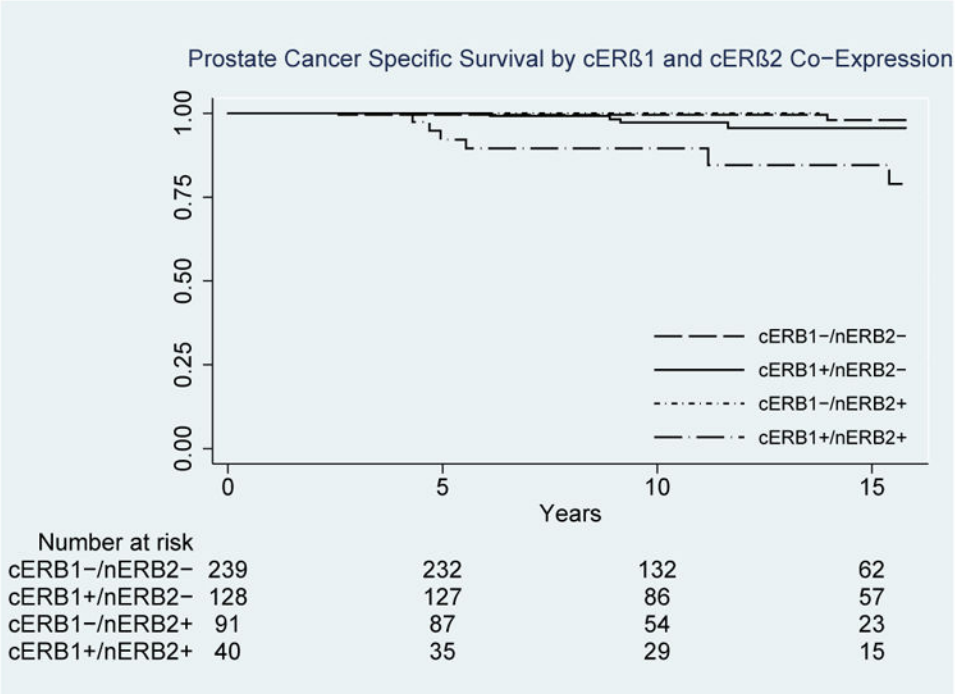

Figure 4.

Prostate cancer-specific survival probability stratified by co-staining status of cERβ1 and nERβ2 staining.

Table 1
Selected characteristics of the prostate cancer patient cohort

Characteristic	N (%)
Age (y) at diagnosis	
35-49	71 (12.5)
50-54	104 (18.4)
55-59	138 (24.4)
60-64	164 (29.0)
65-69	57 (10.1)
70-74	32 (5.6)
Race	
European- American	519 (91.7)
African-American	47 (8.3)
BMI	
<25	180 (31.8)
25-29.9	286 (50.5)
30+	100 (17.7)
Pathological Stage	
Localized	390 (68.9)
Regional	176 (31.1)
Gleason Sum	
2-6	276 (48.8)
7 (3+4)	202 (35.7)
7 (4+3)-10	88 (15.5)
PSA at diagnosis (ng/mL)	
0-3.9	82 (14.5)
4-9.9	338 (59.7)
10-19.9	76 (13.4)
20+	35 (6.2)
Missing	35 (6.2)
Recurrence	
No	341 (60.3)
Yes	119 (21)
Unknown	106 (18.7)
Vital Status	
Alive	495 (87.5)
Prostate cancer death	13 (2.3)
Other cause of death	53 (9.3)
Unknown cause of death	5 (0.9)

Table 2

Association between selected clinicopathologic characteristics and estrogen receptor cERβ1 and nERβ2 staining in prostate cancer patients

	cERβ1				nERβ2			
	None	**Weak**	**Intense**		**None**	**Weak**	**Intense**	
	N (%)	**N (%)**	**N (%)**	**p-value***	**N (%)**	**N (%)**	**N (%)**	**p-value***
	338 (66.4)	**52 (10.2)**	**119 (23.4)**		**387 (74.4)**	**81 (15.6)**	**52 (10.0)**	
Age								
35-49	48 (14.2)	5 (9.6)	10 (8.4)	0.19	49 (12.7)	11 (13.6)	5 (9.6)	0.5
50-54	60 (17.8)	10 (19.2)	26 (21.8)		80 (20.7)	13 (16.1)	5 (9.6)	
55-59	78 (23.1)	20 (38.5)	30 (25.2)		95 (24.5)	19 (23.5)	15 (28.8)	
60-64	94 (27.8)	14 (26.9)	38 (31.9)		105 (27.1)	24 (29.6)	21 (40.4)	
65-69	39 (11.5)	2 (3.8)	8 (6.7)		39 (10.1)	7 (8.6)	4 (7.7)	
70-74	19 (5.6)	1 (1.9)	7 (5.9)		19 (4.9)	7 (8.6)	2 (3.9)	
Race								
European-American	306 (90.5)	51 (98.1)	108 (90.8)	0.19	353 (91.2)	76 (93.8)	47 (90.4)	0.71
African-American	32 (9.5)	1 (1.9)	11 (9.2)		34 (8.8)	5 (6.2)	5 (9.6)	
BMI								
<25	104 (30.8)	16 (30.8)	39 (32.8)	0.61	127 (32.8)	21 (25.9)	16 (30.8)	0.41
25-30	166 (49.1)	27 (51.9)	64(53.8)		185 (47.8)	48 (59.3)	28 (53.8)	
>30	68 (20.1)	9 (17.3)	16 (13.4)		75 (19.4)	12 (14.8)	8 (15.4)	
Stage								
Localized	233 (68.9)	41 (78.8)	71 (59.7)	*0.04*	266 (68.7)	55 (67.9)	30 (57.7)	0.28
Regional	105 (31.1)	11 (21.2)	48 (40.3)		121 (31.3)	26 (32.1)	22 (42.3)	
Gleason Sum								
2-6	165 (48.8)	26 (50.0)	51 (42.9)	0.1	190 (49.1)	38 (46.9)	21(40.4)	0.65
7 (3+4)	130 (38.5)	15(28.8)	42 (35.3)		142 (36.7)	29 (35.8)	20(38.5)	
7 (4+3)-10	43 (12.7)	11 (21.2)	26 (21.8)		55 (14.2)	14 (17.3)	11(21.1)	
PSA at diagnosis (ng/dL)								
0-3.9	54 (16)	7 (13.5)	17 (14.3)	0.21	53 (13.7)	10 (12.3)	13(25.0)	0.38
4-9.9	202 (59.8)	28 (53.8)	68 (57.1)		235 (60.7)	48 (59.3)	25(48.1)	

	cERβ1				nERβ2			
	None	Weak	Intense		None	Weak	Intense	
	N (%)	N (%)	N (%)	p-value*	N (%)	N (%)	N (%)	p-value*
	338 (66.4)	**52 (10.2)**	**119 (23.4)**		**387 (74.4)**	**81 (15.6)**	**52 (10.0)**	
10-19.9	49 (14.5)	8 (15.4)	15 (12.6)		50 (12.9)	13 (16.1)	8 (15.4)	
20+	14 (4.1)	6 (11.5)	12 (10.1)		23 (5.9)	6 (7.4)	4 (7.7)	
Unknown	19 (5.6)	3 (5.8)	7 (5.9)		26 (6.7)	4 (4.9)	2 (3.8)	

* Univariate, Chi^2

Table 3

Multivariable adjusted hazard ratios for prostate cancer recurrence and prostate cancer-specific mortality following radical prostatectomy by estrogen receptor subtype staining intensity

	Recurrence Free Survival		Prostate Cancer-Specific Mortality[$]	
	[*]HR (95% CI)	p-value	[*]HR (95% CI)	p-value
Nuclear ERβ2				
No Staining			Referent	
Weak Staining			0.61 (0.07-5.16)	0.65
Intense Staining			3.89 (1.12-13.42)	*0.03*
Cytoplasmic ERβ1				
No Staining	Referent		Referent	
Weak Staining	0.97 (0.53-1.80)	0.93	1.11 (0.11-11.36)	0.93
Intense Staining	1.72 (1.13-2.62)	*0.01*	6.62 (1.75-24.95)	*0.01*

[*]All models adjusted for age, Gleason sum, pathologic stage and PSA at diagnosis; HR=hazard ratio, CI=confidence interval.

[$]Values represent results from separate Cox proportional hazard models containing clinical factors* and the specified receptor.

Note: Nuclear ERβ2 staining was not significantly associated with recurrence free survival on co-variate analysis and as a result was not included in multivariable analysis.