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HSD17B4 overexpression, an independent biomarker of poor patient outcome in prostate cancer

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ABSTRACT

Steroid hormones and their metabolising enzymes have been studied extensively for their potential role in prostate cancer, with more recent interest in the androgen/estrogen inactivating enzyme 17β-hydroxysteroid dehydrogenase type 4 (HSD17B4). Gene expression profiling showed HSD17B4 to be significantly overexpressed in prostate cancer compared to matched-benign epithelium. We therefore hypothesized that altered HSD17B4 expression may contribute to prostate cancer progression via altered hormone balance. In this study, HSD17B4 mRNA and protein expression were assessed by *in situ* hybridisation (ISH) and immunohistochemistry (IHC), respectively, in tissue arrays of prostate tissue from 172 patients treated by radical prostatectomy. Overexpression of HSD17B4 mRNA and protein was associated with prostate cancer ($P < 0.0001$) and multivariate Cox proportional hazards analysis, adjusted for known prognostic indicators, demonstrated HSD17B4 mRNA and high protein expression were significant independent predictors of poor patient outcome as measured by time until PSA relapse (mRNA: hazards ratio [HR] = 1.90, 95% confidence interval [CI] = 1.15–3.12; $P < 0.0001$; and protein: HR = 2.09, 95% CI = 1.31–3.33; $P = 0.0026$). Here we provide strong evidence that both mRNA and protein overexpression of HSD17B4 is not only associated with the presence of prostate cancer, but is also a significant independent predictor of poor patient outcome.

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1. Introduction

Prostate cancer is the most common malignancy and second-leading cause of cancer deaths among men in most developed countries, including the United States (Jemal et al., 2008) and Australia (The Prostate Cancer Foundation of Australia, www.prostate.org.au/disease.htm). As the average age of the world population increases, so will the burden of prostate cancer on public health systems. Although prostate-specific antigen (PSA) represents the most valuable biomarker for prostate cancer identified to date, limitations include lack of tumour specificity (PSA is organ-specific and not cancer-specific), lack of specificity for early detection, as well as false negative or false positive diagnosis associated with commonly accepted clinical decision limits of PSA levels, the latter resulting in over diagnosis and over treatment (Bunting, 2002; Thompson et al., 2004). Thus there is a clear need for novel biomarkers of prostate cancer, not only for early detection, but also distinguishing indolent from biologically aggressive disease, as well as predicting recurrence after treatment.

It is well established that the sex steroid hormones, androgens and estrogens, acting via steroid receptors and steroid metabolising enzymes, are involved in prostate cancer development and progression (Henderson and Feigelson, 2000), however, the mechanisms underlying these processes are not fully understood. The adrenals provide the prostate with precursor steroids, which are in turn metabolised to active androgens and estrogens via the steroid sulfatases, 3β-hydroxysteroid dehydrogenase (HSD3B), 17β-hydroxysteroid dehydrogenase (HSD17B), 3α-hydroxysteroid

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dehydrogenase (HSD3A), 5α-reductase and aromatase (Soronen et al., 2004). During malignant transformation, changes in expression patterns of these enzymes may significantly change the intracellular steroid content and play a role in the development of prostate cancer (Vihko et al., 2005).

The HSD17Bs are enzymes that regulate both androgen and estrogen action, via oxidation of the C17 alcohol to an inactive or less active ketone, or via reduction of the ketone to an active steroid (dihydrotestosterone, testosterone, or estradiol). Of the fourteen HSD17Bs presently identified, types 2, 4, 8, 10, 11 and 14 are oxidative enzymes, types 1, 3, 5, and 7 and are reductive enzymes, types 12 and 13 have been poorly characterised with regards to steroid metabolism, while types 6 and 9 are absent in humans (Mindnich et al., 2004; Lukacik et al., 2006). The 736 amino acid HSD17B4 (MIM# 601860) polypeptide, also known as D-3-Hydroxylacyl-CoA Dehydrogenase, D-specific Bifunctional Protein (DBP), Multifunctional Protein 2 (MFP-2) or Peroxisomal Multifunctional Enzyme type 2 (MFE-2), is an ancient protein with 80% inter-species amino acid identity and strong conservation of sequence and functionality (Möller and Adamski, 2006). It is the only HSD17B to be localised to the peroxisomes and to display multifunctional properties, including both fatty acid and estradiol oxidation (Leenders et al., 1996; Peltoketo et al., 1999; Baes et al., 2000; Breitling et al., 2001). Its role in steroid metabolism is depicted in Fig. 1. HSD17B4 promotes estrone production, which in turn is metabolised into either non-carcinogenic/marginally carcinogenic 2-OH estrone or carcinogenic 4-OH estrone (or to a lesser extent 16α-OH estrone) metabolites, believed to initiate cancer through the formation of depurinating DNA adducts (Cavalieri et al., 2000, 2006). Along with HSD17B2, HSD17B4 is capable of oxidizing Δ^5-androstenediol (the precursor of testosterone) to dihydroepiandrosterone (DHEA) (Peltoketo et al., 1999).

More recently, interest has developed with regards to the expression of HSD17B4 in prostate cancer. One group reported an increase in HSD17B4 mRNA and protein levels in 4 prostate cancer tissues compared to matched-normal epithelium (Zha et al., 2005), while a second group found HSD17B4 overexpression to be associated with a higher Gleason grade in 23 prostate carcinomas (True et al., 2006). In our laboratory, gene expression profiling confirmed significant HSD17B4 overexpressed in prostate cancer compared to matched-benign epithelium in six patients (supplementary Fig. 1A). Our subsequent mining of published microarray data using the Oncomine 3.0 Cancer Profiling Database (http://www.oncomine.org/main/index.jsp) showed significant (all $P < 0.02$) overexpression of HSD17B4 in prostate cancer compared to normal prostate in 8 out of 15 studies (supplementary Fig. 1B) (Welsh et al., 2001; Dhanasekaran et al., 2001; Luo et al., 2001; LaTulippe et al., 2002; Luo et al., 2002a,b; Singh et al., 2002; Lapointe et al., 2004; Tomlins et al., 2007). Although many published studies have assessed the performance of candidate biomarkers in predicting time to relapse of prostate cancer following radical prostatectomy (reviewed in Quinn et al., 2005), no molecular markers suitable for routine clinical practice that can identify those prostate cancer patients with a high risk of early clinical progression or prostate cancer-specific mortality have been found. Thus we assessed the potential of HSD17B4 as a prognostic marker by determining the level of HSD17B4 transcript and protein expression in a series of 172 post-radical prostatectomy (RP), prostate cancer tissue microarrays (TMAs) using *in situ* hybridisation (ISH) and immunohistochemistry (IHC).

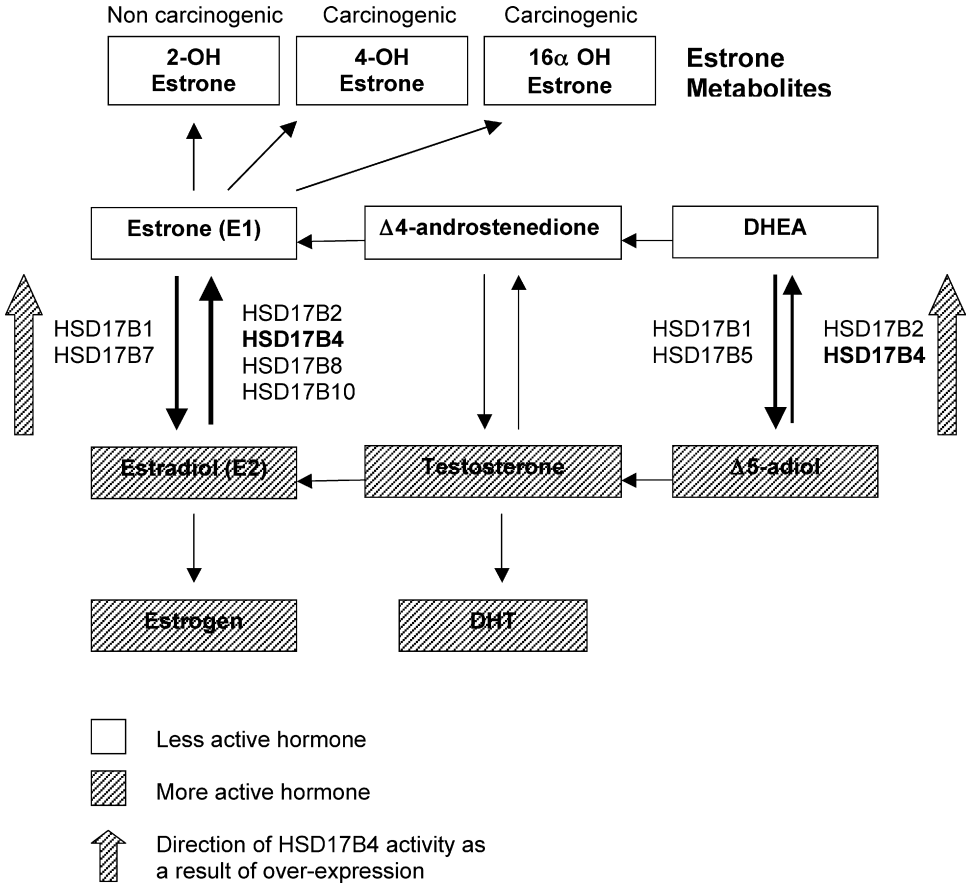

Fig. 1. Steroid metabolism in human prostate tissue. Active hormones (shaded) include DHT, dihydrotestosterone, Δ5-diol, Δ^5-androstenediol and E2, estradiol, while the less active hormones (not shaded) include DHEA, dihydroepiandrosterone and E1, estrone. HSD17B4 overexpression (shaded arrows) will drive the pathway toward hormone inactivation and in turn estrone metabolite production.

2. Materials and methods

2.1. Patient samples

A group of 172 patients of European ancestry, treated with RP for clinically localised prostate cancer at St. Vincent's Hospital Sydney between 1989 and 1997, were consented and included in this study. Patients who received neoadjuvant therapy were excluded from the study. Patients are from a consecutive series and no other exclusionary criteria were used. The median age at RP was 65 years (range: 51–76 years). The demographic and clinical features, including pre-treatment prostate-specific antigen (PSA) levels, pathological stage, and Gleason score, are listed in Table 1. Gleason grading was performed in accordance with established guidelines (Amin et al., 2004; Epstein et al., 2005). The mean follow-up period post-RP was 105.9 months (range: 4.9–188.6 months). Relapse after RP was defined as a rise in serum PSA concentration at or above 0.4 ng/ml rising over a 3-month period or local recurrence on digital rectal examination, confirmed by biopsy or subsequent rise in PSA. Death due to prostate cancer occurred in 1% (2/172) of the patients.

2.2. Tissue microarrays

Archival paraffin-embedded specimens were obtained from the Department of Anatomical Pathology at St. Vincent's Hospital, Douglass Hanly Moir Pathology, or Mayne Pathology (Sydney, Australia). Tissue microarrays (TMAs), containing cores at 1.0 mm in diameter, were prepared with 3–5 samples from each cancer including the dominant and second most dominant Gleason grades, as well as cores from any areas of tertiary grades, as previously described (Kononen et al., 1998; Henshall et al., 2003). All arrays were histologically examined both pre- and post-hematoxylin and eosin staining, and each core verified by two independent pathologists with uropathological expertise. A total of 662 and 632 cores in seven TMAs, representative of 172 patients, were assessed for HSD17B4 riboprobe and antibody staining, respectively.

2.3. Riboprobe

Total RNA was extracted from the PC-3 prostate cancer cell line and template cDNA was synthesised and amplified in a single step using the Titan-One-Step PCR (Roche Diagnostics, Mannheim, Germany) from 100 ng of RNA and the following primers; 3UTR-F: 5′-AGACTACGCCAAGCTCTGA-3′ and 3UTR-R: 5′-TCCTTTCAACTTATCAATGCT-3′ and covering a 324 bp fragment, including 305 bases of the 3′UTR and the last 19 coding bases of the *HSD17B4* gene. Post-purification, the T7 RNA polymerase promoter was ligated to the product using the Lig'nScribe Kit (Ambion, Austin, TX) and amplified using Expand Hi Fidelity (Roche Diagnostics). The identities of the antisense and sense riboprobes were confirmed by direct sequencing. Both riboprobes were labelled with a digoxigenin (DIG) RNA label (Roche Diagnostics). The sense riboprobe was prepared for use as a negative control.

Table 1
Clinicopathological variables in 172 radical prostatectomy patients.

Characteristics	Patients (*N*)
Age (years)	
≤65	89
>65	83
Pre-treatment PSA (ng/ml)	
≤10	79
10.1–20	36
>20	36
ND	5
Clinical stage	
T1A, T1B and T1C	58
T2A, T2B and T2C	107
T3A	7
Pathological stage	
≤pT2C	86
>pT2C	86
Gleason grade	
4–6	102
7	38
8 to 10	30
Surgical margins	
Positive	88
Negative	84
Seminal vesicle involvement	
Positive	29
Negative	143

2.4. In situ hybridisation

Prostate cancer TMAs were cut into 4 μm sections, deparaffinised, hydrated and loaded onto the Ventana Discovery automated instrument (Ventana Medical Systems, Tucson, AZ). Initial processing, formalin-based fixation (30 min at 37 °C) and hydrochloric acid treatment (10 min at 37 °C) was performed using Ventana kits (RiboMap Kit) and reagents (RiboPrep and RiboClear reagent). Following protease digestion (4 min at 37 °C) and denaturation (70 °C for 10 min), the slides were incubated with the antisense or sense riboprobe (0.25 ng/μL) and Ventana RiboHybe hybridisation buffer for 8 h at 65 °C. After a stringency wash (6 min at 60 °C), the second fixation was performed using Ventana RiboFix reagent for 6 min at 37 °C, followed by incubation with anti-digoxin antibody (Sigma Clone D1-22, 1:500) for 30 min. The slides were further incubated with streptavidin-alkaline phosphatase conjugate for 16 min at 37 °C and signal was detected automatically using the Ventana BlueMap NBT/BCIP substrate kit for 6 h at 37 °C. The slides were counterstained with Nuclear Fast Red (DAKO, Glostrup, Denmark) for 90 s prior to mounting. Scoring was performed by a histopathologist (JGK) as "positive" staining (unequivocal blue staining) or no "negative" staining (no blue staining, i.e. only pink counterstaining).

2.5. Immunohistochemistry

Human recombinant GST-HSD17B4 fusion protein was expressed in *E. coli* and affinity-purified on gutathion-sepharose column as previously described (Leenders et al., 1996). The protein revealed in Commassie Blue staining a single band after SDS-PAGE, with an estimated purity of 95%. Approximately 50 mg of GST-HSD17B4 dialysed in phosphate buffer solution was emulsified with Freund's adjuvant and injected both intraperitoneally and subcutaneously into Lou/C rats. After 4 weeks, a final boost was given with 50 mg of protein without adjuvant. Fusion of the myeloma cell line P3X63-Ag8.653 with rat immune spleen cells was performed as described previously (Kremmer et al., 1995). Hybridoma supernatants were tested in a solid-phase immunoassay using HSD17B4 protein absorbed to microtitre plates. The monoclonal antibody used in this study recognizes the C-terminal SCP2-like domain of the human HSD17B4. IHC optimisation was performed on whole prostate cancer sections from RP archival paraffin-embedded specimens and on prostate cancer test TMAs.

Prostate cancer TMAs were cut as 4 μm sections, deparaffinized, rehydrated in graded alcohol (100%, 95% and 70% alcohol). Antigen retrieval was performed in a pressure cooker for 30 s using pH 9.0 Target Retrieval Solution (DAKO, Glostrup, Denmark). Endogenous peroxidases and biotin were blocked with DAKO 3% Hydrogen Peroxide and Biotin Blocking System, respectively. Non-specific binding sites were blocked with DAKO Serum-Free Protein Block for 10 min. The sections, including a positive kidney control section, were incubated with the HSD17B4 antibody for 30 min at room temperature and rinsed with washing buffer. A negative control slide was incubated with rat immunoglobulin G (IgG) at the same concentration as the antibody. The slides were then incubated for 15 min each with biotinylated rabbit anti-rat secondary antibody, followed by detection with Vector ABC Elite (Vector Laboratories, Burlingame, CA). Expression was visualised using DAKO DAB+ for 5 min. The sections were counterstained with Shandons Haematoxylin, dehydrated and mounted for microscopic analysis. The intensity of staining was scored as an ordered categorical variable (none, weak, moderate, or high) by a histopathologist (JGK).

2.6. Statistical analysis

The difference in staining between cancer and benign epithelium was assessed using a logistic mixed model (lmer function in the "Matrix" package in R, www.r-project.org) as follows: logit $P(y_{ij}=1)=\beta_0+U_i+\beta_1\text{Pathol}_{ij}$ where $U_i \sim N(0,\sigma_u{}^2)$, i indexes patient, j indexes measurement, Pathol is an indicator variable (0,1) for benign epithelium versus cancer and y_{ij} is the staining indicator (0,1) for each core. For ISH, y_{ij} equals 1 if the core stains positive and 0 otherwise. For IHC, y_{ij} equals 1 if the core shows high intensity staining and 0 otherwise.

For correlations between HSD17B4 expression and clinical variables, and for survival analyses, dichotomous summary measurements of HSD17B4 staining were derived for each patient. For ISH, a "positive" staining was regarded as staining in one or more cores versus "negative" or no staining. For IHC, "high" staining was determined as an intensity score of high in one or more cores versus "<high" (that is moderate, weak or no staining) in all cores. Associations between HSD17B4 staining and clinicopathological or pathologic variables, were calculated for each patient using the chi-square test for independence (Statview 4.5 Software, Abacus Systems, Berkeley, CA). For survival analysis, disease-specific relapse is defined as a serum PSA concentration of ≥0.2 ng/ml increasing over a 3-month period after surgery.

The primary clinical outcome was defined as time until disease-specific relapse measured from date of RP. Association between HSD17B4 expression and relapse was examined using Kaplan–Meier survival analysis and log-rank test. Both univariate and multivariate analyses were performed using a Cox proportional hazards model for HSD17B4 mRNA and HSD17B4 protein expression status and other known clinical and pathological predictors of outcome, including Gleason grade (4–6, 7, 8–10), preoperative PSA (≤10, 10.1–20, >20 ng/ml), pathological stage (≤pT2C, >pT2C), sur-

gical margin status (positive, negative) and seminal vesicle involvement (positive, negative) with *P* values derived from Likelihood ratio test. Survival analyses were conducted using the "survival" package in R (www.r-project.org). Statistical analysis was two-sided and statistical significance was regarded as $P < 0.05$.

3. Results

3.1. HSD17B4 mRNA and protein expression in prostate cancer

Positive riboprobe and antibody staining using ISH (Fig. 2) and IHC (Fig. 3), respectively, showed epithelial localisation of HSD17B4 mRNA and protein in prostate cancer. For ISH, the sense riboprobe was used as a negative control (Fig. 2D), while kidney tissue (Fig. 3A) and rat IgG (Fig. 3B) were used as IHC positive and negative controls, respectively. The IHC was classified as no (Fig. 3C), weak (Fig. 3D), moderate (Fig. 3E) and high (Fig. 3F) staining intensity. The number of patients with positive ISH staining in one or more cores was 43% (74/170) and for high IHC intensity staining in one or more cores was 29% (50/171). The estimated probability of a prostate cancer core (ISH 0.23 ± 0.02, IHC 0.11 ± 0.02) expressing HSD17B4 was greater than that for adjacent benign epithelium (ISH 0.03 ± 0.01, IHC 0.02 ± 0.01, Fig. 3G). Both ISH and IHC staining confirmed that HSD17B4 expression increased in cancer tissue compared to benign epithelium, with an OR of 9.9 (95% CI 4.5–21.9; $P < 0.0001$) and 5.9 (95% CI 2.8–8.9; $P < 0.0001$), respectively. Gleason Grade was positively correlated with ISH staining but not IHC intensity staining. No other clinicopathological variables were significantly associated with HSD17B4 ISH and IHC staining (Table 2).

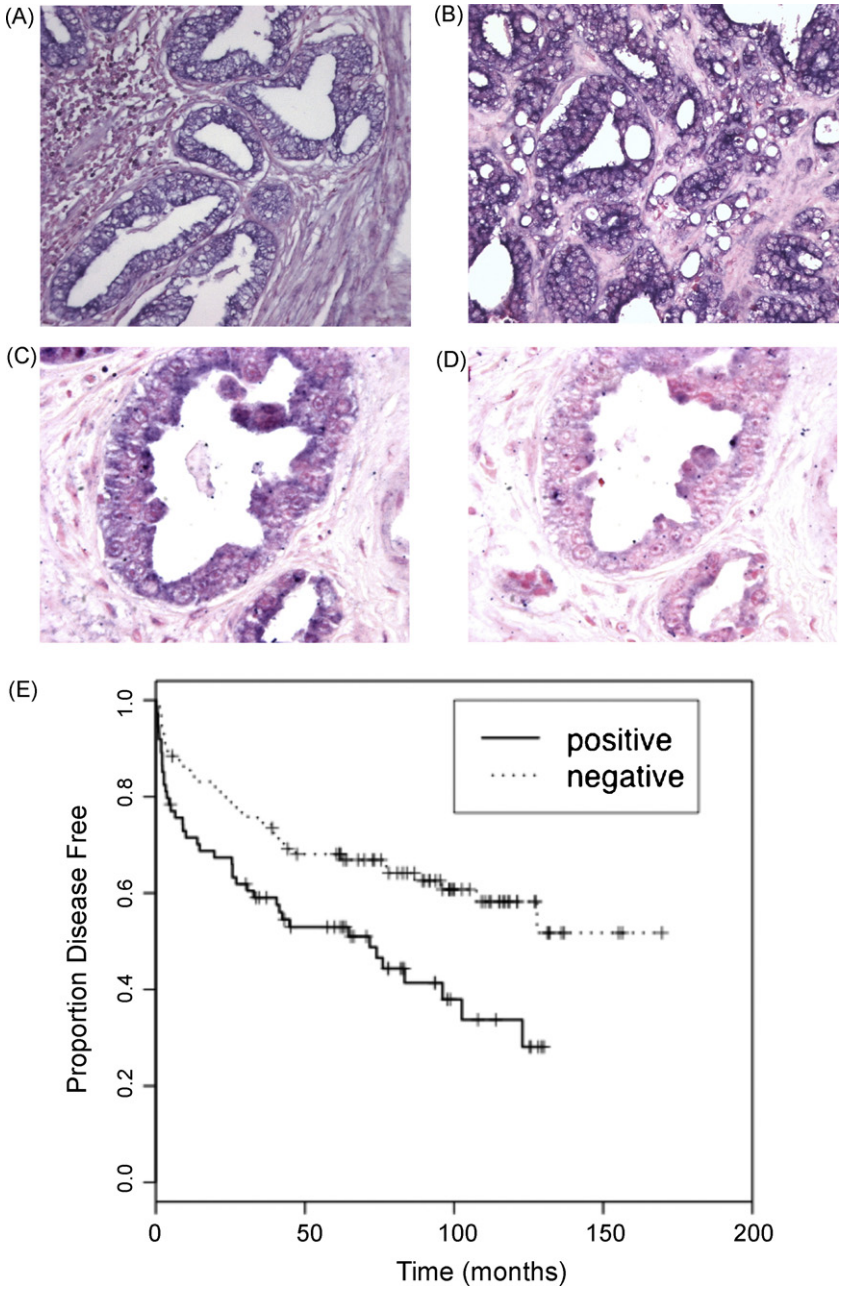

Fig. 2. HSD17B4 *in situ* hybridisation. HSD17B4 antisense riboprobe positive staining (blue-purple) in (A) Gleason pattern 2 (20×), (B) Gleason pattern 3 (20×) and (C) Gleason pattern 4 prostate cancer (400×), while no staining (pink counterstain) is observed for the (D) sense riboprobe (negative control) in Gleason pattern 4 prostate cancer (400×). Epithelial localisation is evident. Kaplan–Meier analysis (E) of patients with "positive" ISH staining in one or more tissue-array cores versus "negative" ISH staining in all cores demonstrates that HSD17B4 mRNA expression is associated with increased risk of relapse.

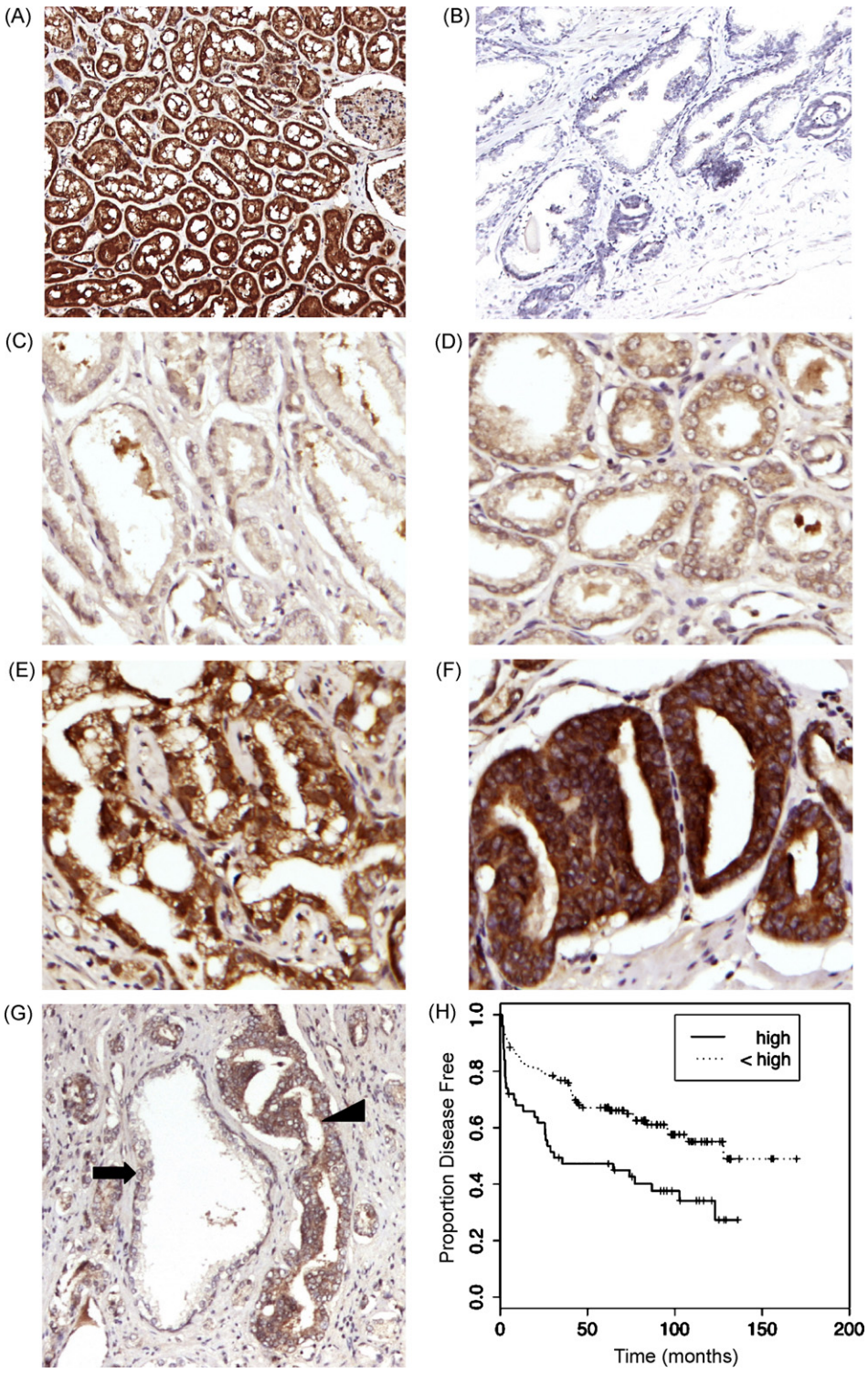

Fig. 3. HSD17B4 immunohistochemistry. HSD17B4 antibody staining (brown) in (A) positive control kidney tissue and no staining (B) in a negative control rat IgG sample. HSD17B4 staining in prostate cancer was epithelial and was scored as (C) no (Gleason pattern 3), (D) weak (Gleason pattern 3), (E) moderate (Gleason pattern 2), and (F) high staining (Gleason pattern 3) on the basis of a qualitative assessment of staining intensity. HSD17B4 was overexpressed in (G) prostate cancer Gleason pattern 3 (black pointer) compared with matched benign epithelium (black arrow). Kaplan–Meier analysis (H) of patients with "high" intensity staining in one or more tissue-array cores versus "<high" intensity staining in all cores, demonstrates that HSD17B4 protein expression is associated with increased risk of relapse.

3.2. HSD17B4 mRNA and protein expression and prostate cancer outcome

Kaplan–Meier survival analysis of patient outcome, defined as time from RP to PSA relapse, demonstrated that both expression of the HSD17B4 mRNA (positive ISH staining in one or more cores) and protein (high IHC intensity in one or more cores) were associated with a significantly worse outcome, logrank $P = 0.004$ (Fig. 2E) and logrank $P = 0.002$ (Fig. 3H), respectively. The IHC staining was also significant when dichotomized for high intensity staining compared with combined no and weak intensity staining (logrank $P = 0.04$) and for high intensity versus moderate intensity staining

Table 2
Correlation between clinicopathological variables and HSD17B4 intensity of staining as measured by ISH or IHC.

Clinicopathological variable	HSD17B4 ISH (positive versus negative) *P*-value*	HSD17B4 IHC (high versus <high) *P*-value*
Pre-treatment PSA (ng/ml)	0.39	0.28
≤10		
10.1–20		
>20		
Pathological stage	0.12	0.77
≤pT2C		
>pT2C		
Gleason grade	**0.0051**	0.76
4–6		
7		
8–10		
Surgical margins	0.89	0.60
Positive		
Negative		
Seminal vesicle involvement	0.80	0.83
Positive		
Negative		

* Chi-square test for independence. $P < 0.05$ was required for statistical significance and is presented in bold.

(logrank $P = 0.003$). Moderate intensity staining was not a significant predictor of outcome (data not shown). Patients lacking high intensity staining (121/172, 71%) showed a significantly better prognosis. Of the patients with positive ISH staining, 44% eluded relapse after 100 months and of the patients with high IHC staining, 37% eluded relapse after 100 months.

In univariate Cox proportional hazards analysis, both HSD17B4 mRNA and protein staining were significant predictors of time to PSA relapse (ISH $P = 0.0051$; HR = 1.89, 95% CI 1.21–2.95 and IHC $P = 0.0035$; HR = 1.99, 95% CI 1.27–3.12). The clinical variables Gleason score ($P < 0.0001$), preoperative PSA ($P < 0.0001$), pathological stage ($P < 0.0001$), surgical margins ($P = 0.012$) and seminal vesicle involvement ($P < 0.0001$), were also significant in univariate Cox proportional hazards analysis.

In multivariable Cox proportional hazards analysis, Gleason score (2–6 versus 7 or 8–10), preoperative PSA (≤10 versus 10.1–20 or >20 (all $P < 0.0001$)) and pathological stage greater than or equal to pT3 versus pT2 (ISH $P = 0.002$; IHC $P = 0.006$), were all predictors of time to relapse, while positive seminal vesicle involvement and surgical margins were significant predictors of relapse in univariate analysis alone. After adjusting for these known clinicopathological prostate cancer prognostic indicators, both HSD17B4 mRNA and protein staining were significant predictors of time to PSA relapse in multivariate analysis (ISH $P < 0.0001$; HR = 1.8, 95% CI 1.1–2.9 and IHC $P = 0.003$; HR = 2.1, 95% CI 1.3–3.3; Table 3). We also note that when IHC intensity was modeled as a continuous variable (none = 0, low = 1, medium = 2, high = 3), IHC intensity was still a significant predictor of outcome in multivariate analysis ($P = 0.03$; HR = 1.4, 95% CI 1.0–1.9).

4. Discussion

Our transcript-profiling data identified HSD17B4 to be 4.7-fold up-regulated in prostate cancer tissue versus matched benign epithelium ($P = 0.002$), while HSD17B types 1, 2, and 3 showed no significant alterations in expression. Another study reported an increase in HSD17B4 mRNA levels in four prostate cancer tissue specimens when compared to matched normal epithelium, while protein levels in these specimens showed a trend towards increased expression in prostate cancer accompanied by an increase in enzymatic activity (Zha et al., 2005). We utilized ISH and IHC to investigate HSD17B4 mRNA and protein expression in a series of prostate cancer TMAs from 172 RP patients, and determined possible associations between levels of expression, clinicopathological parameters and patient outcome.

In this first large-scale study of HSD17B4 expression in prostate cancer, we found both HSD17B4 mRNA and protein to be overexpressed in prostate cancer tissue compared to matched benign epithelium in our TMAs, thus supporting a potential role for HSD17B4 in the development and progression of prostate cancer. In agreement with Takase et al. (2006), we found HSD17B4 expression to be localised to epithelial cells. At the transcript level, HSD17B4 expression correlated with the Gleason Grade of the patient but no such correlation was observed at the protein level. This observation is consistent with that of True et al. (2006), who found that levels of HSD17B4 transcript in individual cores, as measured by RT-PCR, correlated with the Gleason pattern of the cores. Using logistic mixed model, with Gleason pattern as a continuous variable, we also found that ISH of individual cores correlated with Gleason pattern, but IHC intensity staining of individual cores did not correlate with Gleason pattern in our cohort (data not shown).

HSD17B4 overexpression was significantly associated with poor disease outcome, measured as time to PSA relapse. For a

Table 3
Multivariable Cox proportional hazards analysis for clinicopathological variables and either HSD17B4 mRNA (ISH positive versus negative staining) or protein (IHC high versus < high intensity staining) expression in prostate cancer patients having undergone a radical prostatectomy (ISH $n = 163$, IHC $n = 164$).

Variable	ISH (positive versus negative staining)		IHC (high versus <high intensity staining)	
	Hazards ratio (95% CI)	P-value†	Hazards ratio (95% CI)	*P*-value†
HSD17B4 expression	1.90 (1.15–3.12)	**<0.0001**	2.09 (1.31–3.33)	**0.0026**
Gleason score		**<0.0001**		**<0.0000**
2–6	1.00 (Reference)		1.00 (Reference)	
7	2.19 (1.21–3.96)		2.25 (1.24–4.08)	
8–10	1.63 (0.81–3.28)		2.16 (1.14–4.09)	
Preoperative PSA (ng/ml)				
≤10	1.00 (Reference)	**<0.0001**	1.00 (Reference)	**<0.0000**
10.1–20	1.28 (0.73–2.24)		1.40 (0.80–2.43)	
>20	2.29 (1.28–4.10)		2.15 (1.20–3.85)	
Pathological stage (pT3/4 versus pT2)	2.70 (1.46–5.01)	**0.0014**	2.28 (1.26–4.10)	**0.0058**
Surgical margins (positive versus negative)	0.81 (0.46–1.42)	0.46	0.85 (0.49–1.46)	0.56
Seminal vesicle involvement (positive versus negative)	1.24 (0.67–2.29)	0.49	1.33 (0.72–2.45)	0.36

† $P < 0.05$ was required for statistical significance and is presented in bold.

prognostic marker to be of potential clinical utility it must be measured in the context of known predictors of prostate cancer recurrence and death, including Gleason score, preoperative serum PSA concentration, pathological stage, seminal vesicle involvement or surgical margin involvement (Quinn et al., 2005). In this study all variables were significant predictors of outcome in univariate analysis (data not shown). In multivariate analysis, all except surgical margin and seminal vesicle involvement were significant predictors. When adjusting for traditional prognostic indicators, HSD17B4 mRNA ($P < 0.0001$) and protein ($P = 0.0026$) overexpression remained strong independent predictors of outcome. We note that the vast majority of the Australian cohort is Caucasian so the results may not be able to be generalised to other groups. It is clear the HSD17B4 could not be used to completely replace the current clinical markers, but we recommend independent validation utilizing contemporary prostate cancer series to further investigate the potential role of HSD17B4 immunostaining as a molecular marker of prostate cancer outcome.

The potential mechanism of involvement of HSD17B4 in the biology of prostate cancer may be as a direct result of its role as a steroid-metabolizing enzyme and carcinogenic metabolite production. Androgens in turn regulate a number of genes including genes involved in lipid (fatty acid and cholesterol) metabolism (Swinnen et al., 1997a,b). HSD17B4 is also actively involved in the β-oxidation of branched chain fatty acids in the peroxisomes via the non-inducible pathway (Adamski and Jakob, 2001), while upregulation of fatty acid synthase, specifically α-methylacyl-CoA racemase (AMACR), has been observed in multiple cancers including prostate cancer (Luo et al., 2002a,b). AMACR, required for the catalytic conversion of R-α-methylacyl-CoA esters to their S-stereoisomers prior to HSD17B4 oxidation, displays increased enzymatic activity in prostate cancer (Zha et al., 2003). Branched chain fatty acids originate almost exclusively from the diet, particularly dairy products and red meat, both of which have been associated with increased risk of prostate cancer (Chan et al., 2001). In addition, peroxisomal β-oxidation generates hydrogen peroxide a potential source of carcinogenic oxidative damage (Tamatani et al., 1999). These studies therefore implicate not only the steroid-metabolizing pathway, but also the fatty acid β-oxidation pathway in prostate cancer development. In light of these and our findings, further studies are required to elucidate the potential biological role of these pathways and HSD17B4 in prostate cancer.

This study further contributes to a potential role of the steroid metabolising and fatty acid oxidation enzyme HSD17B4 in prostate cancer and was the first study to investigate its involvement in disease prognosis. We demonstrate that HSD17B4 is overexpressed in prostate cancer and that this overexpression is a predictor of poor patient outcome. The implication of this finding will require further assessment of HSD17B4 as a prostate cancer prognostic marker and as a candidate for therapeutics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2008.11.021.

References

Adamski, J., Jakob, F.J., 2001. A guide to 17β-hydroxysteroid dehydrogenases. Mol. Cell. Endocrinol. 171, 1–4.
Amin, M.B., Grignon, D.J., Humphrey, P.A., Srigley, J.R., 2004. Gleason Grading of Prostate Cancer. Lippincott Williams and Wilkins, Philadelphia.
Baes, M., Huyghe, S., Carmeliet, P., Declercq, P.E., Collen, D., Mannaerts, G.P., Van Veldhoven, P.P., 2000. Inactivation of the peroxisomal multifunctional protein-2 in mice impedes the degradation of not only 2-methyl-branched fatty acids and bile acid intermediates but also of very long chain fatty acids. J. Biol. Chem. 275, 16329–16336.
Breitling, R., Marijanovic, Z., Perovic, D., Adamski, J., 2001. Evolution of 17β-HSD type 4, a multifunctional protein of β-oxidation. Mol. Cell. Endocrinol. 171, 205–210.
Bunting, P.S., 2002. Screening for prostate cancer with prostate-specific antigen: beware of biases. Clin. Chim. Acta 315, 71–97.
Cavalieri, E., Frenkel, K., Liehr, J.G., Rogan, E., Roy, D., 2000. Estrogens as endogenous genotoxic agents—DNA adducts and mutations. J. Natl. Cancer. Inst. Monogr. 27, 75–93.
Cavalieri, E., Chakravarti, D., Guttenplan, J., Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan, E., Russo, J., Santen, R., Sutter, T., 2006. Catechol estrogen quinones as inhibitors of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochim. Biophys. Acta 1766, 63–78.
Chan, J.M., Stampfer, M.J., Ma, J., Gann, P.H., Gaziano, J.M., Giovannucci, E.L., 2001. Dairy products, calcium, and prostate cancer risk in the Physicians' Health Study. Am. J. Clin. Nutr. 74, 549–554.
Dhanasekaran, S.M., Barrette, T.R., Ghosh, D., Shah, R., Varambally, S., Kurachi, K., Pienta, K.J., Rubin, M.A., Chinnaiyan, A.M., 2001. Delineation of prognostic biomarkers in prostate cancer. Nature 412, 822–826.
Epstein, J.I., Allsbrook Jr., W.C., Amin, M.B., Egevad, L.L., 2005. ISUP Grading Committee. The 2005 International society of urological pathology (ISUP) consensus conference on Gleason grading of prostatic cancer. Am. J. Surg. Pathol. 29, 1228–1242.
Henderson, B.E., Feigelson, H.S., 2000. Hormonal carcinogenesis. Carcinogen 21, 427–433.
Henshall, S.M., Afar, D.E.H., Hiller, J., Horvath, L.G., Quinn, D.I., Rasiah, K.K., Gish, K., Willhite, D., Kench, J.G., Gardiner-Garden, M., Stricker, P.D., Scher, H.I., Grygiel, J.J., Agus, D.B., Mack, D.H., Sutherland, R.L., 2003. Survival analysis of genome-wide gene expression profiles of prostate cancers identifies new prognostic targets of disease relapse. Cancer Res. 63, 4196–4203.
Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., Thun, M.J., 2008. Cancer statistics, 2008. CA Cancer J. Clin. 58, 71–96.
Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M.J., Sauter, G., Kallioniemi, O.P., 1998. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat. Med. 4, 844–847.
Kremmer, E., Kranz, B.R., Hille, A., Klein, K., Eulitz, M., Hoffmann-Fezer, G., Feiden, W., Herrmann, K., Delecluse, H.J., Delsol, G., Bornkamm, G.W., Mueller-Lantzsch, N., Grässert, F.A., 1995. Rat monoclonal antibodies differentiating between the Epstein–Barr virus nuclear antigen 2A (EBNA2A) und 2B (EBNA2B). Virology 208, 336–342.
Lapointe, J., Li, C., Higgins, J.P., van de Rijn, M., Bair, E., Montgomery, K., Ferrari, M., Egevad, L., Rayford, W., Bergerheim, U., Ekman, P., DeMarzo, A.M., Tibshirani, R., Botstein, D., Brown, P.O., Brooks, J.D., Pollack, J.R., 2004. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc. Natl. Acad. Sci. U.S.A. 101, 811–816.
LaTulippe, E., Satagopan, J., Smith, A., Scher, H., Scardino, P., Reuter, V., Gerald, W.L., 2002. Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. Cancer Res. 62, 4499–4506.
Leenders, F., Tesdorpf, J.G., Markus, M., Engel, T., Seedorf, U., Adamski, J., 1996. Porcine 80-kDa protein reveals intrinsic 17 beta-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase, and sterol transfer activities. J. Biol. Chem. 271, 5438–5442.
Lukacik, P., Kavanagh, K.L., Oppermann, U., 2006. Structure and function of human 17β-hydroxysteroid dehydrogenases. Mol. Cell. Endocrinol. 248, 61–71.
Luo, J.H., Duggan, D.J., Chen, Y., Sauvageot, J., Ewing, C.M., Bittner, M.L., Trent, J.M., Isaacs, W.B., 2001. Human prostate cancer and benign prostatic hyper-

plasia: molecular dissection by gene expression profiling. Cancer Res. 61, 4683–4688.
Luo, J.H., Yu, Y.P., Cieply, K., Lin, F., Deflavia, P., Dhir, R., Finkelstein, S., Michalopoulos, G., Becich, M., 2002a. Gene expression analysis of prostate cancers. Mol. Carcinog. 33, 25–35.
Luo, J., Zha, S., Gage, W.R., Dunn, T.A., Hicks, J.L., Bennett, C.J., Ewing, C.M., Platz, E.A., Ferdinandusse, S., Wanders, R.J., Trent, J.M., Isaacs, W.B., De Marzo, A.M., 2002b. α-Methylacyl-CoA racemase: a new molecular marker for prostate cancer. Cancer Res. 62, 2220–2226.
Mindnich, R., Möller, G., Adamski, J., 2004. The role of 17 beta-hydroxysteroid dehydrogenases. Mol. Cell. Endocrinol. 218, 7–20.
Möller, G., Adamski, J., 2006. Multifunctionality of human 17beta-hydroxysteroid dehydrogenases. Mol. Cell. Endocrinol. 248, 47–55.
Peltoketo, H., Luu-The, V., Simard, J., Adamski, J., 1999. 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family: nomenclature and main characteristics of the 17HSD/KSR enzymes. J. Mol. Endocrinol. 23, 1–11.
Quinn, D.I., Henshall, S.M., Sutherland, R.L., 2005. Molecular markers of prostate cancer outcome. Eur. J. Cancer 41, 858–887.
Singh, D., Febbo, P.G., Ross, K., Jackson, D.G., Manola, J., Ladd, C., Tamayo, P., Renshaw, A.A., D'Amico, A.V., Richie, J.P., Lander, E.S., Loda, M., Kantoff, P.W., Golub, T.R., Sellers, W.R., 2002. Gene expression correlates of clinical prostate cancer behavior. Cancer Cell 1, 203–209.
Soronen, P., Laiti, M., Törn, S., Härkönen, P., Patrikainen, L., Li, Y., Pulkka, A., Kurkela, R., Herrala, A., Kaija, H., Isomaa, V., Vihko, P., 2004. Sex steroid hormone metabolism and prostate cancer. J. Steroid. Biochem. Mol. Biol. 92, 281–286.
Swinnen, J.V., Ulrix, W., Heyns, W., Verhoeven, G., 1997a. Coordinate regulation of lipogenic gene expression by androgens: evidence for a cascade mechanism involving sterol regulatory element binding proteins. Proc. Natl. Acad. Sci. U.S.A. 94, 12975–12980.
Swinnen, J.V., Esquenet, M., Goossens, K., Heyns, W., Verhoeven, G., 1997b. Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. Cancer Res. 57, 1086–1090.
Takase, Y., Lévesque, M.-H., Luu-The, V., El-Alfy, M., Labrie, F., Pelletier, G., 2006. Expression of enzymes involved in estrogen metabolism in human prostate. J. Histochem. Cytochem. 54, 911–921.
Tamatani, T., Hattori, K., Nakashiro, K., Hayashi, Y., Wu, S., Klumpp, D., Reddy, J.K., Oyasu, R., 1999. Neoplastic conversion of human urothelial cells in vitro by overexpression of H_2O_2-generating peroxisomal fatty acyl CoA oxidase. Int. J. Oncol. 15, 743–749.
Thompson, I.M., Pauler, D.K., Goodman, P.J., Tangen, C.M., Lucia, M.S., Parnes, H.L., Minasian, L.M., Ford, L.G., Lippman, S.M., Crawford, E.D., Crowley, J.J., Coltman Jr., C.A., 2004. Prevalence of prostate cancer among men with a prostate-specific antigen level < or = 4.0 ng per millilitre. N. Engl. J. Med. 350, 2239–2246.
Tomlins, S.A., Mehra, R., Rhodes, D.R., Cao, X., Wang, L., Dhanasekaran, S.M., Kalyana-Sundaram, S., Wei, J.T., Rubin, M.A., Pienta, K.J., Shah, R.B., Chinnaiyan, A.M., 2007. Integrative molecular concept modeling of prostate cancer progression. Nat. Genet. 39, 41–51.
True, L., Coleman, I., Hawley, S., Huang, C.Y., Gifford, D., Coleman, R., Beer, T.M., Gelmann, E., Datta, M., Mostaghel, E., Knudsen, B., Lange, P., Vessella, R., Lin, D., Hood, L., Nelson, P.S., 2006. A molecular correlate to the Gleason grading system for prostate adenocarcinoma. Proc. Natl. Acad. Sci. U.S.A. 103, 10991–10996.
Vihko, P., Herrala, A., Härkönen, P., Isomaa, V., Kaija, H., Kurkela, R., Li, Y., Patrikainen, L., Pulkka, A., Soronen, P., Törn, S., 2005. Enzymes as modulators in malignant transformation. J. Steroid Biochem. Mol. Biol. 93, 277–283.
Welsh, J.B., Sapinoso, L.M., Su, A.I., Kern, S.G., Wang-Rodriguez, J., Moskaluk, C.A., Frierson, H.F., Hampton, G.M., 2001. Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. Cancer Res. 61, 5974–5978.
Zha, S., Ferdinandusse, S., Denis, S., Wanders, R.J., Ewing, C.M., Luo, J., De Marzo, A.M., Isaacs, W.B., 2003. Alpha-methylacyl-CoA racemase as an androgen independent growth modifier in prostate cancer. Cancer Res. 63, 7365–7376.
Zha, S., Ferdinandusse, S., Hicks, J.L., Denis, S., Dunn, T.A., Wanders, R.J., Luo, J., De Marzo, A.M., Isaacs, W.B., 2005. Peroxisomal branched chain fatty acid β-oxidation pathway is upregulated in prostate cancer. Prostate 63, 316–323.