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IGFBP-3 Nuclear Localization Predicts Human Prostate Cancer Recurrence

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Abstract Insulin-like growth factor binding protein-3 (IGFBP-3) is a pro-apoptotic, anti-metastasic, and anti-angiogenic protein. Low serum IGFBP-3 has been associated with risk of more aggressive prostate cancer (PCa). We investigated the impact of nuclear and cytoplasmic IGFBP-3 protein expression levels in PCa by examining their in situ expression across a wide spectrum of primary tumors by immunohistochemical analysis of tissue microarrays. Immunohistochemistry was performed on PCa microarrays constructed from 226 hormone naïve patients who underwent radical prostatectomy. Both cytoplasmic and nuclear IGFBP-3 expressions were scored in a semi-quantitative fashion using an integrated measure of intensity and positivity. The distribution of IGFBP-3 protein expression was examined across the spectrum of epithelial tissues, and its association with standard clinicopathological covariates and tumor recurrence was examined. There was a broad range of IGFBP-3 staining across all histologies examined. Tumor had higher IGFBP-3 cytoplasmic and nuclear staining than benign histologies. For IGFBP-3 nuclear staining, PCa was significantly different than benign prostatic hyperplasia, normal prostate, and prostate intraepithelial neoplasia. As both a continuous and dichotomized variable, higher *nuclear* IGFBP-3 expression had statistically significant associations with PCa recurrence. The *cytoplasmic* staining had no significance in any patient subgroup. In patients with low-grade cancer, IGFBP-3 nuclear positivity was a better predictor of recurrence than baseline PSA, tumor margin status, TNM tumor stage, or presence of capsular invasion. High nuclear IGFBP-3 is amongst the strongest predictors of cancer recurrence in patients with low-grade prostate cancers and may therefore play an important role in risk stratification.

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Abbreviations

ABC	Avidin–biotin complex
BPH	Benign prostatic hyperplasia
H & E	Hematoxylin and eosin
IGFBP-3	Insulin-like growth factor binding protein-3
IHC	Immunohistochemistry
PIN	Prostatic intraepithelial neoplasia
PCa	Prostate cancer
PSA	Prostate-specific antigen
TMA	Tissue microarray

Introduction

Despite gradual improvements in annual mortality rates, prostate cancer (PCa) remains the leading malignancy in men (excluding basal and squamous cell skin cancers), with

over 240,000 new cases estimated for 2011 [22]. Tremendous variability in the clinical behavior of PCa, even within categories defined by traditional clinical and pathologic means of risk stratification, has accelerated interest in the identification of molecular biomarkers of PCa progression. Optimally, early detection and analysis of these biomarkers could accurately stratify patients for appropriate surveillance and risk-adapted therapy. Currently utilized prognostic factors include Gleason grade, tumor stage, and preoperative serum PSA [2, 17, 33]. A recent study identified a Tri-Marker Proliferation Index (proliferation signature genes: MKI67 (Ki-67; also a classic proliferation biomarker), TOP2A (DNA topoisomerase II, alpha), and E2F1 (E2F transcription factor 1)) as a superior prognostic tool in the prediction of PCa recurrence [27]. Clinical use of some of these proliferation markers is already underway in other hormone-responsive malignancies such as breast [7] and gastroentero-pancreatic neuroendocrine tumors [21].

Insulin-like growth factor binding protein (IGFBP)-3 is a potent inducer of apoptosis in PCa (for review see [37]). In addition, anti-angiogenic [26, 29] and anti-metastatic properties have been described in vitro and in vivo [28, 29]. Many of its effects, including inhibition of the NF-κB pathway, appear to be independent of its ability to sequester the IGFs [16]. Epidemiologic studies indicate that higher serum IGF-1 and lower IGFBP-3 levels are independently associated with a greater risk of prostate cancer [3, 4]. Moreover, circulating IGFBP-3 levels were independent predictors of PCa progression in multivariate models that include PSA, biopsy Gleason score, and clinical stage [34].

In a small early study [15], it was reported that IGFBP-3 immunostaining in prostate cancer tissue did not correlate with the clinical outcome of patients undergoing radical prostatectomy. However, a subsequent study performed only in Japanese men demonstrated that intratumoral IGFBP-3 expression in post-neoadjuvant hormonal therapy (NHT) specimens was a useful predictive marker of biochemical recurrence [30]. To further assess the potential clinical significance of IGFBP-3 expression in human PCa, we utilized our large tissue microarray (TMA) platform to examine the association of IGFBP-3 protein-level expression with prostate tissue histology and cancer recurrence. Here, we report for the first time that high nuclear IGFBP-3 is associated with an increased risk of prostate cancer recurrence after radical retropubic prostatectomy.

Methods

Prostate Tissue Microarray Patients

The study cohort consisted of 226 randomly selected, hormone naïve patients who underwent radical retropubic prostatectomy

between 1984 and 1995. All prostate tumors were staged according to the 1997 American Joint Committee on Cancer TNM staging system [11] and histologically graded by a single experienced GU pathologist (DS) using the Gleason scoring system [14]. All cases were of the histological type "adenocarcinoma, conventional, not otherwise specified" [38]. Of the 226, 194 were informative for both recurrence outcomes and marker expression data. The median age at the time of surgery was 65 (range 46 to 76). One hundred fourteen (59 %) patients were low grade (Gleason score 2–6); 80 (41 %) were high grade (Gleason score 7–10). Thirty-three patients (17 %) had seminal vesicle invasion (pT3b). Concurrent regional lymphadenectomy accompanied 192 (99 %) cases, of which 10 (5 %) were positive for metastases. One hundred thirty-two (68 %) patients were margin negative and 62 (32 %) were margin positive. Regarding capsular invasion, 43 (22 %) had no invasion, 112 (58 %) had invasion, and 39 (20 %) had extracapsular extension. In 101 cases (52 %), the tumors were confined to the prostate (organ confined here=T2a or T2b with negative lymph nodes, no capsular extension and with negative surgical margins). Thirty-seven (19 %) patients were considered high risk based on seminal vesicle and/or nodal positivity. The maximum preoperative serum PSA was known for 174 patients (90 %), with a median value of 9.3 ng/ml (range 0.6–96.5). Table 1 shows the clinicopathologic data for this cohort.

A retrospective analysis for outcome assessment was based on detailed, anonymized clinicopathologic information linked to the TMA tissue specimens. Recurrence, defined as a postoperative serum PSA of 0.2 ng/ml or greater, was seen in 67 (35 %) patients. Median follow-up, defined as the time from surgery to recurrence or to last contact in non-recurring patients, was 49.0 months (range 0.1–163) for the entire cohort.

TMA Construction

Formalin-fixed, paraffin-embedded archival tumor specimens were obtained from the UCLA Department of Pathology under IRB approval. Case material was reviewed for tissue array construction by a study pathologist (DS). At least three core tissue biopsies (each 0.6 mm in diameter) were taken from morphologically representative regions of each prostate tumor and precisely arrayed as previously described [23]. Tumor samples were accompanied by matching benign (morphologically normal or hypertrophic) and in situ neoplastic lesions (PIN), when available. For staining, standard non-adhesive sections (5 μm) were transferred to glass slides using an adhesive slide system (Instrumedics Inc., Richmond, IL) to support cohesion of the array elements.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed using a G column-purified hybridoma-derived mouse monoclonal

Table 1 Relationship of IGFBP-3 protein expression with clinicopathologic parameters of patients with prostate adenocarcinomas

All patients	Number	Mean percent nuclear IGFBP-3 expression (standard error)	P value[a] nuclear IGFBP-3 (continuous variable)	"Low" nuclear IGFBP-3 positivity<9 % (percent of total)	"High" nuclear IGFBP-3 positivity≥9 % (percent of total)	P value[b] nuclear IGFBP-3 (dichotomized variable)
Total cases	194	9.4 (0.99)		123 (63)	71 (37)	
Age at surgery			0.57 (NS) (Spearman)			0.074 (NS)[a]
Median (range)	65 (46–76)			64.0 (46–76)	66.0 (50–75)	
Mean	63.7			63.2	64.6	
Gleason score			0.32 (NS)			0.88 (NS)
2–6	114 (59)	9.14 (1.31)		73 (59)	41 (58)	
7–10	80 (41)	9.82 (1.53)		50 (41)	30 (42)	
Pathology pT stage[c]			>0.99 (NS)			0.24 (NS)
PT2–pT3a	161 (83)	9.25 (1.04)		99 (80)	62 (87)	
PT3b	33 (17)	10.26 (2.95)		24 (20)	9 (13)	
Lymph node status	(n=192)		0.93 (NS)			0.75 (NS)
Positive	10 (5)	8.63 (3.68)		7 (6)	3 (4)	
Negative	182 (95)	9.55 (1.04)		114 (94)	68 (96)	
Surgical margins			0.11 (NS)			0.025
Positive	62 (32)	11.56 (1.87)		32 (26)	30 (42)	
Negative	132 (68)	8.41 (1.16)		91 (74)	41 (58)	
Capsular			0.33 (NS)[d]			0.21 (NS)
Involvement			0.15 (NS) (Spearman)			
No Invasion	43 (22)	7.20 (1.46)		28 (23)	15 (21)	
Invasion	112 (58)	9.33 (1.35)		75 (61)	37 (52)	
Extension	39 (20)	12.11 (2.59)		20 (16)	19 (27)	
Organ confined[e]	(n=192)		0.025			0.10 (NS)
Yes	101 (52)	7.70 (1.22)		70 (57)	31 (44)	
No	93 (48)	11.29 (1.57)		53 (43)	40 (56)	
High risk[f]	(n=192)		0.83 (NS)			0.19 (NS)
Yes	37 (19)	9.89 (2.69)		27 (22)	10 (14)	
No	155 (81)	9.40 (1.07)		94 (78)	61 (86)	
PreOpPSA ng/ml	(n=174)		0.74 (NS) (Spearman)			0.86 (NS)[a]
Median (range)	9.3 (0.6–96.5)			9.9 (1.3–96.5)	8.5 (0.6–60.7)	
Mean	14.0			13.9	14.0	
<10	92 (53)	8.96 (1.24)	0.80 (NS)	55 (50)	37 (58)	0.35 (NS)

Table 1 (continued)

All patients	Number	Mean percent nuclear IGFBP-3 expression (standard error)	P value[a] nuclear IGFBP-3 (continuous variable)	"Low" nuclear IGFBP-3 positivity<9% (percent of total)	"High" nuclear IGFBP-3 positivity≥9% (percent of total)	P value[b] nuclear IGFBP-3 (dichotomized variable)
≥10	82 (47)	9.62 (1.70)		55 (50)	27 (42)	
Recurrence[g]			0.0039[h]			0.0033[h]
Yes	67 (35)	13.64 (2.09)		33 (27)	34 (48)	
No	127 (65)	7.19 (0.99)		90 (73)	37 (52)	
Total follow-up[i], (mo)			0.28 (NS) (Spearman)			0.28 (NS)[a]
Median (range)	49.0 (0.1–163.0)			49.0 (0.1–163.0)	52.0 (1.0–1,117.0)	
Mean	52.8			55.5	48.1	

[a] P value was determined by the Mann–Whitney U test unless otherwise specified. P values are corrected for ties

[b] P value was determined by the Pearson chi-squared test with Yates continuity correction unless otherwise specified. Fisher's Exact used for 2×2 table

[c] pT3b indicates seminal vesicle invasion. There are no pT4 cases

[d] P value was determined by the Kruskal–Wallis test. Capsular involvement is also examined as a continuous variable using the Spearman correlation, P value corrected for ties

[e] Organ confined=no capsular extension and/or seminal vesicle and/or lymph node involvement. Margins are negative

[f] High risk=seminal vesicle and/or nodal positivity

[g] Recurrence=PSA elevation raising>0.2 ng/ml status post-radical prostatectomy

[h] IGFBP-3 mean positivity association with recurrence by logistic regression of continuous data; (P=0.0039, hazard 1.03; 95 % confidence interval 1.01–1.06), and of dichotomized data (P=0.0033; 2.51; 95 % confidence interval 1.36–4.63). IGFBP-3 expression was the independent variable

[i] Total follow-up=time to recurrence or last follow-up in non-recurrence

anti-IGFBP3 antibody (R&D Systems MAB305; clone 84728; IgG2b). A standard two-step indirect avidin–biotin complex (ABC) method was used (Vectastain Elite PK-6102, Vector Laboratories, Burlingame, CA) for visualization. Following deparaffinization in xylenes, the array sections were rehydrated in graded alcohols. The sections were placed in 0.01 M sodium citrate buffer (pH6.0) and heated in a pressure cooker for antigen retrieval. Endogenous peroxidase was quenched with 3 % hydrogen peroxide in methanol at room temperature for 10 min, and then the non-specific protein binding was blocked with 5 % normal goat serum for 60 min. Avidin and biotin were sequentially applied to block endogenous biotin binding sites. The primary antibody was then applied at 10 μg/ml final concentration and incubated at 4 °C overnight. Biotinylated goat anti-mouse IgG was then applied for 60 min at room temperature. The ABC complex was applied for 45 min followed by the chromogen diaminobenzidine (DAB) for 1 min. PBS (10 mM, pH7.4) was used for all wash steps and dilutions. Incubations were performed in a humidity chamber. The sections were counterstained with hematoxylin, followed by dehydration and mounting. As a negative assay control, non-immune mouse IgG2b antibody (Dako X0944) was applied at the same concentration as the IGFBP-3 antibody.

Scoring of Immunohistochemistry

Semi-quantitative assessment of antibody on the TMAs was performed by a single study pathologist (HY) blinded to the clinicopathological variables. The target tissue for scoring was the glandular prostatic epithelium; scoring of benign tissues did not include basal cells. Tissue spot histology and grading were re-confirmed on the counterstained study slides. IGFBP-3 cytoplasmic expression was scored using two measures, intensity on a 0–3 scale (0=negative, 1=weakly positive, 2=moderately positive, 3=strongly positive), and percentage of positively stained target cells (range 0–100 % positive) staining at each intensity. To better represent overall protein levels, we combined the frequency and intensity measures into an integrated intensity measure using the following formula: ((percent staining at intensity 3*3)+(percent staining at intensity 2*2)+(percent staining at intensity 1*1))/100. IGFBP-3 nuclear expression was scored noting the percentage of positively stained target cells (range 0–100 % positive). To represent expression within individual cases, the mean pooled integrated intensity (cytoplasmic) and positivity (nuclear) of the invasive tumor spots was used for outcome assessment.

Statistical Analysis

The Kruskal–Wallis and Mann–Whitney U tests were used to determine the significance of IGFBP-3 protein expression

differences between nominal clinicopathologic prognostic variables. Associations of IGFBP-3 expression with continuous covariates were tested with the Spearman correlation. We used the Pearson chi-squared test to examine the association of dichotomized IGFBP-3 expression groups versus nominal variables. Recurrence was defined as a rising total prostate-specific antigen (PSA)>0.2 ng/ml status post prostatectomy, and time to recurrence was calculated from the date of the primary surgery. Patients without recurrence at last follow-up were censored. Kaplan–Meier plots were used to visualize recurrence-free time distributions, and the log–rank test was used to test for differences between them. To assess which covariates associate with recurrence-free time, we fit both univariate and multivariate Cox Proportional Hazards regression models [18]. All P values were two sided and $P<0.05$ was considered significant. All statistical analyses were performed using R statistical software (http://www.r-project.org/) and StatView version 5 (SAS Institute Inc., Cary, N.C., USA).

Results

IGFBP-3 Subcellular Localization by Histological Category

In human prostate tissue, staining of IGFBP-3 was ubiquitous but highly variable across prostate tissues (Fig. 1). Invasive tumor glands are shown displaying negative to weak (Fig. 1a) and moderate to strong (Fig. 1b) diffuse cytoplasmic staining (both cases lacking nuclear staining), whereas a nuclear-predominant staining pattern is seen in the glands of case C (Fig. 1c). While individual cases tend to express a homogenous pattern, rare cases demonstrate distinct regional cytoplasmic or nuclear expression patterns (Fig. 1d; tumor glands in upper and lower tissue spot regions with cytoplasmic- and nuclear-predominant expression, respectively).

We examined the IGFBP-3 protein expression distribution stratified by histological category (Fig. 2). All 226 hormone naive cases provided epithelium-informative microarray spots; 197 of those included informative spots from cancer tissues. The intensity of IGFBP-3 protein expression in cells staining by immunohistochemistry, as seen in 1,123 informative primary tissue microarray spots containing benign prostatic hyperplasia (BPH; $n=114$), morphologically normal prostate (NL; $n=276$), prostatic intraepithelial neoplasia (PIN; $n=38$) and invasive prostate cancer (PCa; $n=695$), are shown as mean bar graphs (Fig. 2a).

In general, tumor had higher IGFBP-3 cytoplasmic and nuclear staining than benign histologies. For cytoplasmic staining, all t test comparisons were $P<0.0001$ except between PIN and PCa ($P=0.56$). For nuclear staining, PCa was significantly different than BPH ($P<0.0001$), NL ($P<0.0001$), and PIN ($P=0.0021$) (Fig. 2b).
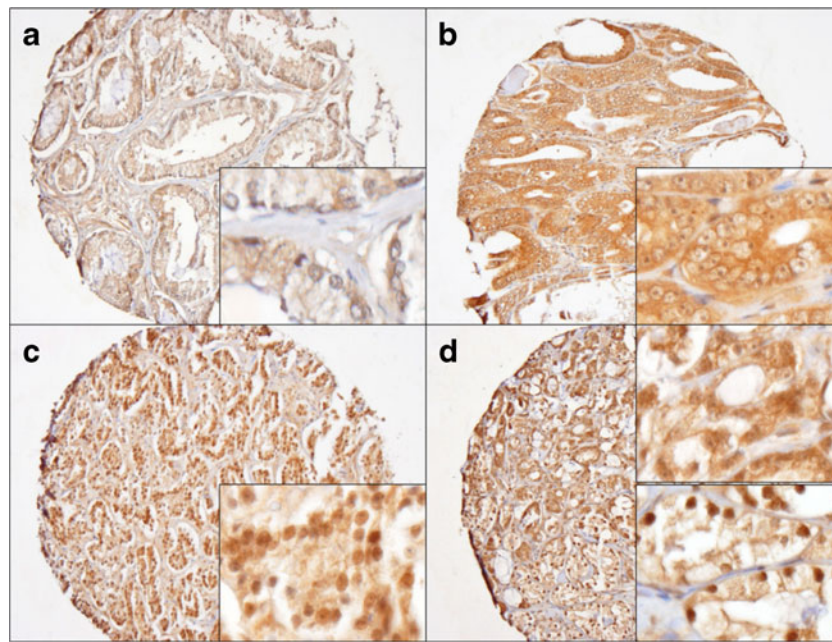
Fig. 1 Histological staining patterns of IGFBP-3 protein on human prostate tissue microarrays. A variety of immunohistochemical staining patterns for IGFBP-3 protein are shown in a representative sampling of glandular human prostate tissues. The tissue spots are all heavily invested with invasive adenocarcinoma and the protein content is depicted by the brown chromagen deposition. Invasive tumor glands are shown displaying negative to weak (**a**) and moderate to strong (**b**) diffuse cytoplasmic staining (both cases lacking nuclear staining), whereas a nuclear-predominant staining pattern is seen in the glands of case **c**. While individual cases tend to express a homogenous pattern, rare cases demonstrate distinct regional cytoplasmic or nuclear expression patterns (**d**; tumor glands in upper and lower tissue spot regions with cytoplasmic- and nuclear-predominant expression, respectively) (100× magnification with 400× inserts)

There was a broad range of tumor IGFBP-3 *cytoplasmic* staining in cases which approximated a normal curve (Fig. 3a). However, *nuclear* staining (0–100 %) was much lower in expression (Fig. 3b) and, when positive, was only occasionally >20 % positive in cells (only seven cases went higher than 50 % pos, max at 70 %). A similar pattern was seen in low- versus high-grade groupings (data not shown). When mean tumor expression (pooled tumor spot data) was examined, a weak but statistically significant inverse correlation between cytoplasmic and nuclear IGFBP-3 expression was found (data not shown). Correlation analysis to cytoplasmic expression was hampered because the nuclear
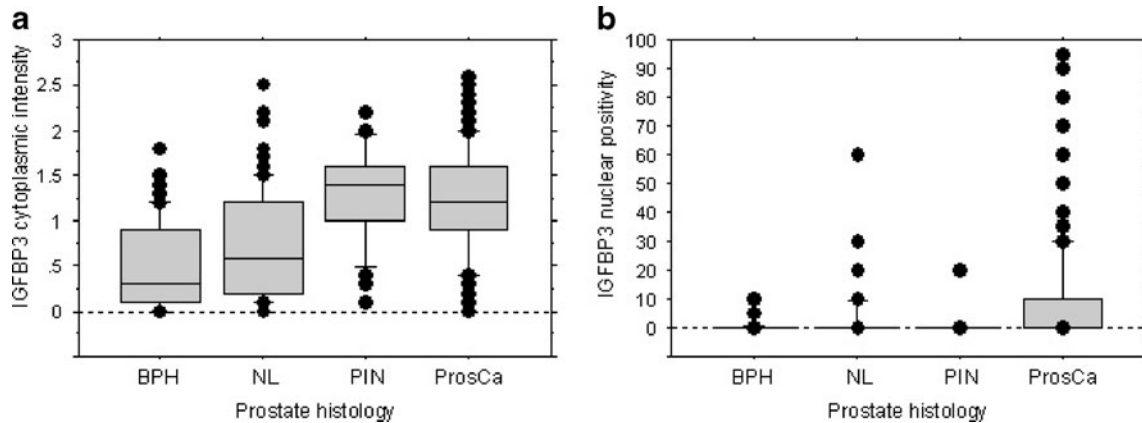

Fig. 2 IGFBP-3 protein expression stratified by histological category. The distribution of IGFBP-3 protein expression measured by immuno-histochemistry and stratified by tissue microarray spot histology is shown in **a** and **b** (cytoplasmic intensity [0–3], and nuclear positivity [0–100 %], respectively). The *box plot* gives the median observation (*box dividing line*, where visible), the 25th to 75th percentile range (*box range*), 10th and 90th percentile (*whiskers*) and <10th and >90th percentile observations (*dots*). Represented are 114 benign prostatic hyperplasia (BPH), 276 morphologically normal (NL), 38 prostatic intraepithelial neoplasia (PIN), and 695 cancer (PCa) samples, comprising a total of 1,123 informative tissue microarray spots. The mean IGFBP-3 cytoplasmic expression is significantly different comparing all histology group pairs ($P<0.0001$, unpaired t test), except PCa versus PIN ($P=0.56$). The median nuclear expression in PCa is significantly different versus BPH or NL (both $P<0.0001$), and versus PIN ($P=0.0021$), and no other comparisons are significant
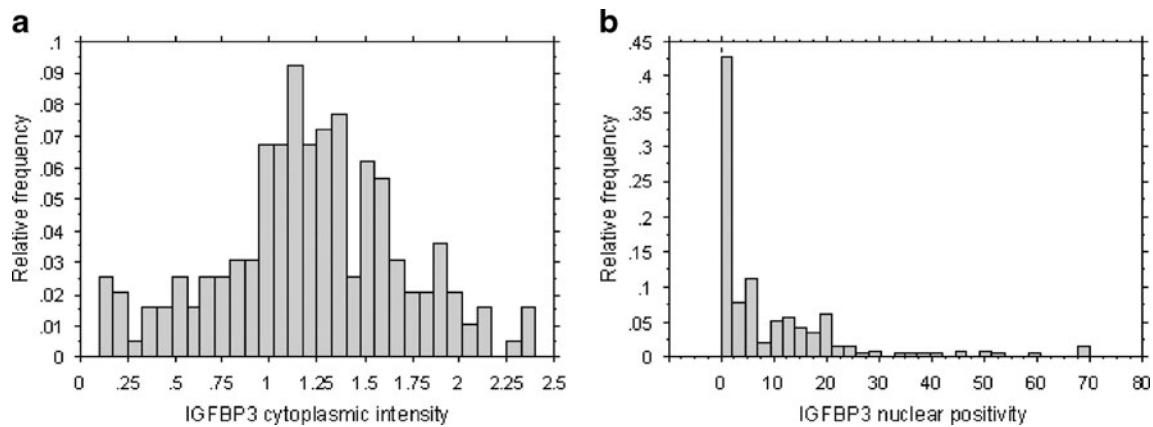
Fig. 3 IGFBP-3 protein expression distribution across prostate cancer cases by tissue microarray. The immunohistochemical distribution of IGFBP-3 protein expression is shown for 194 informative primary prostate cancer cases. The relative frequency of expression is stratified by mean cytoplasmic intensity (0–3) and mean nuclear positivity (0–100 %), in **a** and **b**, respectively

expression component was limited to a small number of cases.

Nuclear IGFBP-3 Protein Expression is a Strong Predictor of Prostate Cancer Recurrence

We next examined the potential association of IGFBP-3 protein expression with tumor recurrence following radical prostatectomy. Recurrence data was available for 194 IGFBP-3 informative cases (114 low grade and 80 high grade). Case-level expression was derived by pooling the mean integrated intensities of the spots, as previously reported [13]. Supervised survival tree analysis was applied to pooled data, and a dichotomized population was defined with an optimal cutpoint of 10 % mean integrated intensity representing individuals with higher versus lower nuclear IGFBP-3 expression. We examined the association of IGFBP-3 as both a continuous and a dichotomized variable with established prognostic factors, and found that expression of nuclear IGFBP-3 was associated with disease recurrence (Table 2). Figure 4a shows a Kaplan–Meier estimate of cancer recurrence-free time stratified by nuclear IGFBP-3 expression for all cases. Significantly, the median recurrence-free time was 60 months for cases with high nuclear IGFBP-3, and the median was not reached for cases with low nuclear IGFBP-3 (log–rank $P=0.0074$). Moreover, in patients with primary low-grade cancer, the presence of nuclear IGFBP-3 was even more predictive of tumor recurrence than in "all cases" (Fig. 4b; log–rank $P=0.0007$). In the low-grade group, the median recurrence-free time was 100 months for patients with nuclear IGFBP-3, compared to the median not being reached for patients without nuclear IGFBP-3. Cox proportional hazards analyses were done using established prognostic factors to determine which variables were significant as independent predictors of time to PSA recurrence (Table 2). Of particular note is the

strength of nuclear IGFBP-3 predictive power as a dichotomized variable, which remained an independent predictor in multivariate analysis. As an independent predictor, it is close to other conventional clinicopathologic parameters such as stage, especially in primary low-grade cancer.

Cut-point analysis for cytoplasmic staining was attempted (all possible cutpoints in all scoring measures [intensity, positivity and integrated values, each pooled using the mean and median value], in all cases and also the low- and high-grade groups). No statistically significant cutpoint was found. Likewise, cytoplasmic expression used as a continuous value was not statistically significant for any scoring measure or patient strata.

Discussion

Conventional clinicopathological variables serve as important predictors of prostate cancer patient outcomes, but do not elucidate the molecular mechanisms required for cancer cell survival nor suggest therapeutic targets for its treatment. Currently, biopsy Gleason grade together with stage, serum prostate specific antigen (PSA) and tumor volume are used to carefully select men with low risk PCa that will likely benefit from treatment other than radical prostatectomy (RP) such as active surveillance or low dose brachytherapy [1, 8]. However, many men with low grade PCa at biopsy will later be found to have been misclassified, and to later be found to have been diagnosed with high-grade PCa at RP. Therefore, RP Gleason score is better than biopsy Gleason score as an indicator of biochemical recurrence and poor clinical outcome [6, 12, 17, 19], suggesting that Gleason sum upgrading could be clinically relevant to estimate the probability of a more aggressive variant of PCa [10]. A recent study suggests that determination of preoperative IGFBP-3 circulating levels could be useful to predict Gleason score

Table 2 Relationship of IGFBP-3 protein expression with clinicopathologic parameters for patients with low Gleason score (2–6) prostate adenocarcinomas

Low grade (Gleason score 2–6)	Number	Mean percent nuclear IGFBP-3 expression (standard error)	P value[a] nuclear IGFBP-3 (continuous variable)	"Low" nuclear IGFBP-3 positivity<9 % (percent of total)	"High" nuclear IGFBP-3 positivity≥9 % (percent of total)	P value[b] nuclear IGFBP-3 (dichotomized variable)
Total cases	114	9.1 (1.31)		73 (64)	41 (36)	
Age at surgery			0.83 (NS) (Spearman)			0.37 (NS)[a]
Median (range)	64 (46–75)			64.0 (46–75)	65.0 (50–73)	
Mean	63.4			63.0	64.1	
Pathology pT stage[c]			0.64 (NS)			>0.99 (NS)
PT2–pT3a	109 (96)	8.81 (1.28)		70 (96)	39 (95)	
PT3b	5 (4)	16.33 (11.33)		3 (4)	2 (5)	
Lymph node status	(n=112)		0.32 (NS)			>0.99 (NS)
Positive	2 (2)	13.75 (11.25)		1 (1)	1 (2)	
Negative	110 (98)	9.19 (1.35)		70 (99)	40 (98)	
Surgical margins			0.15 (NS)			0.021
Positive	27 (24)	10.38 (2.11)		12 (16)	15 (37)	
Negative	87 (76)	8.75 (1.60)		61 (84)	26 (63)	
Capsular involvement			0.11 (NS)[d] 0.048 (Spearman)			0.25 (NS)
No invasion	32 (28)	6.59 (1.79)		22 (30)	10 (24)	
Invasion	63 (55)	8.43 (1.63)		42 (58)	21 (51)	
Extension	19 (17)	15.79 (4.70)		9 (12)	10 (24)	
Organ confined[e]			0.006			0.007
Yes	75 (66)	7.21 (1.46)		55 (75)	20 (49)	
No	39 (34)	12.85 (2.53)		18 (25)	21 (51)	
High risk[f]	(n=112)		0.36 (NS)			0.71 (NS)
Yes	7 (6)	15.60 (8.21)		4 (6)	3 (7)	
No	105 (94)	8.52 (1.32)		67 (94)	38 (93)	
PreOp PSA (ng/ml)	(n=105)		0.93 (NS) (Spearman)			0.89 (NS)[a]
Median (range)	7.9 (0.6–56.0)			8.2 (1.9–49.2)	7.9 (0.6–56.0)	
Mean	10.6			10.3	11.2	
<10	66 (63)	8.14 (1.26)		40 (60)	26 (68)	0.41 (NS)

Table 2 (continued)

Low grade (Gleason score 2–6)

	Number	Mean percent nuclear IGFBP-3 expression (standard error)	P value[a] nuclear IGFBP-3 (continuous variable)	"Low" nuclear IGFBP-3 positivity<9 (percent of total)	"High" nuclear IGFBP-3 positivity≥9% (percent of total)	P value[b] nuclear IGFBP-3 (dichotomized variable)
≥10	39 (37)	9.72 (2.74)		27 (40)	12 (32)	
Recurrence[g]			0.0007[h]			0.001[h]
Yes	22 (19)	20.51 (4.36)		7 (10)	15 (37)	
No	92 (81)	6.42 (1.09)		66 (90)	26 (63)	
Total follow-up[i] (mo)			0.27 (NS) (Spearman)			0.20 (NS)[a]
Median (range)	60.0 (0.1–163.0)			65.0 (0.1–163.0)	58.0 (2.0–117.0)	
Mean	60.4			64.2	53.7	

[a] P value was determined by the Mann–Whitney U test unless otherwise specified. P values are corrected for ties

[b] P value was determined by the Pearson chi-squared test with Yates continuity correction unless otherwise specified. Fisher's Exact used for 2×2 table

[c] pT3b indicates seminal vesicle invasion. There are no pT4 cases

[d] P value was determined by the Kruskal–Wallis test. Capsular involvement is also examined as a continuous variable using the Spearman correlation, P value corrected for ties

[e] Organ confined=no capsular extension and/or seminal vesicle and/or lymph node involvement. Margins are negative

[f] High risk=seminal vesicle and/or nodal positivity

[g] Recurrence=PSA elevation raising>0.2 ng/ml status post-radical prostatectomy

[h] IGFBP-3 mean positivity association with recurrence by logistic regression of continuous data; (P=0.0007, hazard 1.03, 95 % confidence interval 1.03–1.10), and of dichotomized data (P=0.001; 5.44; 95 % confidence interval 1.99–14.87). IGFBP-3 expression was the independent variable

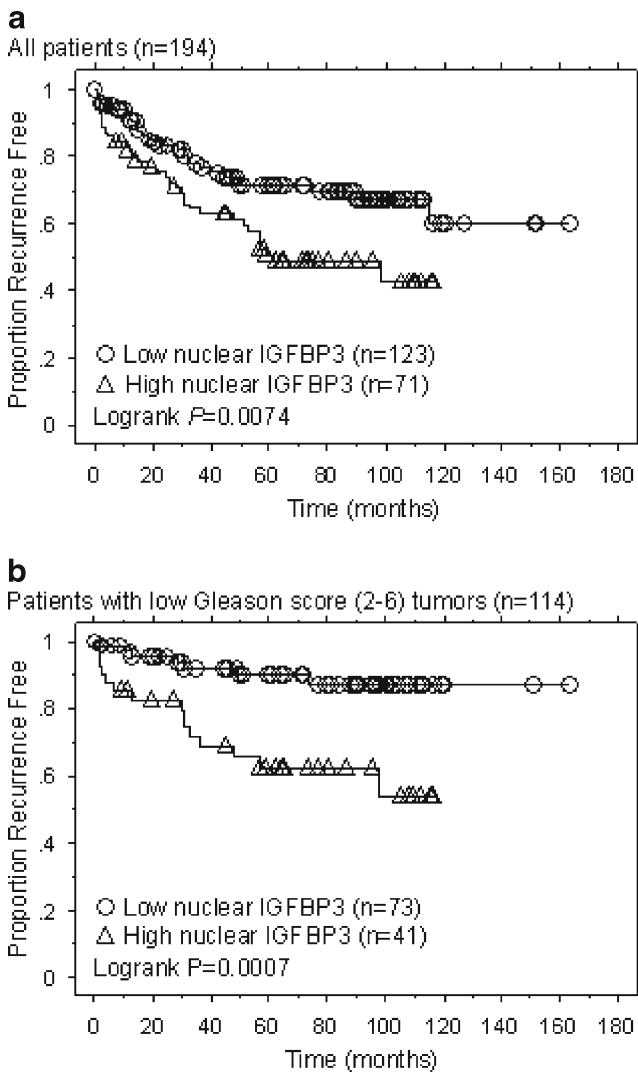[i] Total follow-up=time to recurrence or last follow-up in non-recurrence

Fig. 4 Kaplan–Meier curves for time to prostate cancer recurrence. Kaplan–Meier curves for time to tumor recurrence stratified by nuclear IGFBP-3 protein expression status are seen in all patients, **a** (*n*=194), and in patients stratified by low tumor grade, (Gleason score range 2–6), **b** (*n*=114). An IGFBP-3 nuclear positivity of ≥9 % and <9 % are considered "High" and "Low" IGFBP-3, respectively. A high nuclear expression phenotype is associated with a higher risk of developing recurrent prostate cancer in both patient cohorts (all patients, *P*=0.0074; low-grade cases only, *P*=0.0007). Censored times marked by *circles* and *triangles*

Described mechanisms involved in tumor suppression include: induction of senescence [32]; inhibition of angiogenesis [26]; activation of a novel death receptor [20]; internalization via endocytic pathways and nuclear localization [24]; and mitochondrial translocation of RXRα: Nur77 with activation of the intrinsic pathway of apoptosis [25, 31]. We have recently characterized a nuclear export sequence in IGFBP-3 and have demonstrated that mutation of this sequence and sequestration of IGFBP-3 in the nucleus abrogates its apoptosis-inducing properties [31]. This is consistent with our current observation that predominantly nuclear IGFBP-3 staining in PCa tumors (and presumably deficient in ability to undergo IGFBP-3 dependent apoptosis) predict poor outcome as identified by PSA recurrence. We hypothesize that specific post-translational modifications (PTM) and/or protein partners determine nuclear sequestration and inactivation of IGFBP-3 in the progression of PCa. Clearly more studies are needed to elucidate the mechanisms of nuclear sequestration.

An early study pre-TMA methodology assessed IGFBP-3 presence in human PCa in a small number of specimens using standard immunohistochemical (IHC) techniques. Additionally, 24 patients with a preoperative diagnosis of clinically localized prostate adenocarcinoma with 5-year follow-up information were compared with nine normal prostates from organ donors or from patients undergoing cystoprostatectomy. Malignant transformation of prostatic epithelium was associated with a significant decrease in the amount of immunoreactive IGFBP-3; however, this parameter did not correlate with Gleason grade of the tumor or with patient outcome [15]. The presence/absence of predominantly nuclear IGFBP-3, however, was not mentioned in this paper which was also limited by its small sample size.

Similar IHC techniques were used in another study of 42 patients who underwent neoadjuvant hormonal therapy (NHT) and RP [30]. Pre-NHT and post-NHT specimens were examined for expression of IGFBP-3 and apoptosis. Examination of the representative IHC figure reveals that NHT resulted in a significant increase in IGFBP-3 expression in both nuclear and cytoplasmic compartments. This increase in expression correlated with the induction of apoptosis. Patients with high IGFBP-3 expression in the post-NHT specimens had a good prognosis. In these specimens, presumably IGFBP-3 was able to exit the nucleus to induce apoptosis.

Our clinical dataset lacks post-surgical treatment information so that IGFBP-3 expression levels or subcellular localization cannot be linked to future radiotherapy or drug response. Therefore, our study does not directly implicate IGFBP-3 expression levels and sensitivity to subsequent treatment in our cohort and future studies would benefit from inclusion of this variable. In addition, all of the surgical materials are derived from primary prostatectomies.

upgrading alone and/or in combination with biopsy T-stage and biopsy Gleason score [36]. These findings correlate well with previous studies showing that circulating IGFBP-3 levels were lowest in patients with bony metastases and lower in patients with metastases to regional lymph nodes than in patients with non-metastatic PCa or in healthy subjects [34]. In addition, local expression of IGFBP-3 has been inversely associated with Gleason score [9].

Tumor suppression by IGFBP-3 in PCa has been characterized in vitro and in vivo, and this suppression can be mediated by IGF-independent mechanisms [5, 35].

Additional validation studies in patients with hormone refractory recurrence and distant metastases may shed light on IGFBP-3 expression and subcellular localization in these advanced patient groups.

Prostate cancer remains a disease with heterogeneous outcomes, encompassing a wide spectrum of patients from indolent cancer that may not require treatment to patients with highly aggressive cancers for whom treatment is ineffective. Therefore, informative biomarkers are urgently needed to guide both patient surveillance and clinical intervention. This study reports for the first time that the high nuclear expression of IGFBP-3 in primary human prostate cancers is associated with poor outcomes, in both all patients combined, and also for the subset of prostate cancer patients having only low-grade disease. Our study, therefore, identifies nuclear localization of IGFBP-3 (a surrogate of intrinsic pathway activation) as a potential new prognostic marker in PCa and identifies a potential "escape mechanism" (nuclear sequestration) for cancer cell progression. While the value of circulating IGFBP-3 levels remain controversial as a predictive factor for prostate cancer, our study establishes intratumoral IGFBP-3 expression, and specifically subcellular localization, as an important predictive factor for prostate cancer recurrence. As additional controversies exist over the ideal treatment algorithm for low-grade PCa, nuclear sequestration of IGFBP-3 may emerge as a prognostic tool to aid in selecting individual patients for early intervention of PCa.

Conflict of interest statement   The authors declare that they have no conflict of interest.

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