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Expression of phosphatase of regenerating liver (PRL)-3, is independently associated with biochemical failure, clinical failure and death in prostate cancer

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Abstract

Background

Prostate cancer (PC) stratification needs new prognostic tools to reduce overtreatment. Phosphatase of regenerating liver (PRL-3) is a phosphatase found at high levels in several cancer types, where its expression is associated with survival. A recent PC cell line study has shown it to be involved in PC growth and migration.

Methods

We used a monoclonal antibody to evaluate the expression of PRL-3 in PC tissue of patients in an unselected cohort of 535 prostatectomy patients. We analyzed associations between PRL-3 expression and biochemical failure-free survival (BFFS), clinical failure-free survival (CFFS) and PC death-free survival (PCDFS).

Results

Cytoplasmic PRL-3 staining in tumor cells was significantly correlated to expression of molecules in the VEGFR-axis, but not to the clinicopathological variables. High PRL-3 was not significantly associated with survival in the univariate analysis for BFFS (p = 0.131), but significantly associated with CFFS (p = 0.044) and PCDFS (p = 0.041). In multivariate analysis for the various end points, PRL-3 came out as an independent and significant indicator of poor survival for BFFS (HR = 1.53, CI95% 1.10–2.13, p = 0.012), CFFS

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(HR = 2.41, CI95% 1.17–4.98, p = 0.017) and PCDFS (HR = 3.99, CI95% 1.21–13.1, p = 0.023).

Conclusions

PRL-3 is independently associated with all PC endpoints in this study. Since high PRL-3 expression also correlates with poor prognosis in other cancers and functional studies in PC support these findings, PRL-3 emerges as a potential treatment target in PC.

Introduction

Prostate cancer (PC) is the fourth most common cancer overall and the second most common in men worldwide [1]. Presently, the identification of clinically relevant PC is challenging since overdiagnosis and overtreatment coexist, while many die of aggressive PC [2]. There are ongoing efforts to improve the identification of aggressive PC, but these efforts are hampered by the lack of useful tools. Although recent efforts, like the composite pre-biopsy STHLM3 model, are entering the field[3], the morphology grade scored by pathologists is still today the strongest predictor of aggressive disease[4]. Besides, there is currently no widely used prognostic molecular tissue markers in PC. Hence, improved prognostic and more so predictive molecular markers are urgently needed in this field.

Phosphatase of regenerating liver (PRL)-3 is a dual specificity phosphatase with ability to dephosphorylate tyrosine, serine and threonine residues. In 2001, Vogelstein's group suggested that the PRL-3 gene (gene name: *PTP4A3*) is important for colorectal cancer metastasis as they found high levels of *PTP4A3* expression in metastases from colorectal cancer compared to non-metastatic tumors and normal colorectal epithelium[5]. Studies have found PRL-3 to be associated with epithelial-mesenchymal transition (EMT) and cancer progression[6]. Other studies have shown PRL-3 to be associated with metastatic potential and poor prognosis in a large number of cancers[7–16], as well as being upregulated in myeloma cells[17]. Due to these studies, PRL-3 has been proposed a promising biomarker for assessing tumor aggressiveness and metastatic potential[18]. In addition, targeting of PRL-3 has been proposed and several studies have recently reported endogenous suppressing proteins[19] and a new humanized antibody against PRL3 (PRL3-zumab) has been tested in orthotopic gastric tumors[20].

In PC, PRL-3 has previously been identified as a mediator of PC progression and aggressiveness in an integrated assessment of aggressiveness through gene copy number and gene expression analyses[21]. As PRL-3 is a potential cancer biomarker and biomarkers in PC are in high demand, Exploring the expression and biological role of PRL-3 in PC cells, Vandsemb et al [22] found PRL-3 mRNA to be highly expressed in PC tissue compared to benign prostate tissue, and the PRL-3 protein was expressed in both primary PCs and regional lymphatic metastasis. Further *in vitro* studies found inhibition to induce growth arrest and decreased migration of PC cancer cells. They also evaluated and found PRL-3 expression in 4/4 cases by immunohistochemistry.

To further explore PRL-3's role in PC, we aimed to elucidate the expression profile and prognostic impact of PRL-3 in a large cohort of PC patients. Herein, we present the results using a validated PRL-3 antibody on tissue microarrays (TMAs) from a large, well described retrospective cohort with an extensive follow-up[23].

Material and methods

Patients, tissue micro arrays and endpoints

Patients were included after retrospective identification of 671 patients from the archives of the departments of pathology in two health regions in Norway, undergoing radical prostatectomy (RP) for adenocarcinoma of the prostate between 01.01.1995 to 31.12.2005. One-hundred and thirty-one (131) patients were excluded, due to non-available tissue blocks for re-evaluation (St. Olav n = 112, NLSH n = 3, UNN n = 15) [23]. A total of 535 eligible patients with available tissues and complete follow-up data were included in this retrospective cohort study. Two-hundred and twenty-eight (228) patients were from St. Olav Hospital/Trondheim University Hospital (St. Olav) in the Central Norway region, and 59 from Nordlandssykehuset Bodo (NLSH) and 248 from the University Hospital of North Norway (UNN), both in the Northern Norway region. In total, 435 patients were submitted to open retropubic resection and 100 patients had perineal resection.

From the cohort we constructed 12 tissue micro array (TMA) blocks. A tissue-arraying instrument (Beecher Instruments, Silver Springs, MD, USA) was used to harvest cores from formalin-fixed paraffin-embedded (FFPE) tissue blocks from included patients. Two cores were sampled from the most dedifferentiated neoplastic cell compartment, hereafter designated *tumor*. Furthermore, two cores were sampled from reactive tumor stroma, hereafter designated *stroma*. The cores were carefully inserted into paraffin blocks. Then, 4 μm sections were cut by a Micron microtome (HM355S) and affixed to glass slides prior to immunostaining and scoring.

Biochemical failure (BF) was defined as a PSA $\geq$ 0.4 ng/ml and BF-free survival (BFFS) was calculated as time from surgery to last follow up (FU) date or date with PSA $\geq$ 0.4 ng/ml. Clinical failure (CF) was defined as symptomatic, locally advanced progression or radiologically verified metastasis to bone, visceral organs or lymph nodes. Clinical failure-free survival (CFFS) was calculated as time from surgery to last fFU date without CF or to date of CF. Last follow-up update was December 2015, and calculated median follow-up of survivors was 150 months.

For more extensive information regarding patients, exclusion, definitions of variables and endpoints, see our previous report[23].

Immunohistochemistry

TMA paraffin block sections slides were dried overnight at 37˚C. PRL-3 immunohistochemical staining of the cut sections was performed using the Ventana Discovery ULTRA autostainer (Tucson, Arizona, USA). After paraffin embedded tissues were dewaxed, antigen retrieval was applied using Ventana ULTRA Cell Conditioning-1 (CC1) for 32 minutes at 95˚C. Endogenous peroxidase was blocked by discovery inhibitor CM (#760–4306, Ventana) for 12 minutes. Sections were incubated with non-commercial mouse monoclonal antibody[24, 25] (kind gift from professor Qi Zeng, Agency for Science, Technology and Research (ASTAR), Singapore) with 1/50 dilution for 32 minutes at 36˚C. As secondary antibody, OmniMap anti-mouse HRP (#760–4310, Ventana) was loaded for 20 minutes, followed by 8 minutes of HRP amplification. The detection chromogen was ChromoMap DAB (#760–159; Ventana). Counterstaining was performed using the hematoxylin II (#790–2208, Ventana) counterstain for 32 minutes and then with a bluing reagent for 8 minutes. Staining was performed in one single experiment and a human multiple organ (normal and malignant) tissue array was included for specificity control of antibody. Normal tonsil and liver adenocarcinoma were used as negative and positive tissue controls, respectively.

Scoring of immunohistochemistry and cut-offs

PRL-3 expression was scored semiquantitatively. We initially explored the expression with our dedicated uropathologist (E.R.), and agreed on scoring definitions and scales. Then, two scorers (E.R, M.R) performed all scoring and reported the scores independently of each other. We sought to assess expression in applicable compartments (tumor, non-malignant epithelium and stroma) and different cell compartments (cytoplasmic, nuclear or membranous). Scorable PRL-3 expression was only possible where positivity was present in more than a minor subset. We ended up with the following scoring scale based on observed expression: A. Tumor cytoplasmic cell intensity on a four-tier scale (0 = negative, 1 = weak, 2 = intermediate, 3 = strong), and B. Tumor nuclear density on a four tier scale (0 = 0%, 1 = 0–5%, 2 = 5–50% and 3 >50% of nuclear tumor cells stained). A cut-off of 1.5 was defined for all analyses.

Statistical analyses

We used the SPSS software version 23 (IBM SPSS Inc., Chicago, IL, USA) for statistical analyses. For the Inter-observer reliability of scoring, we used the two-way random effect model with absolute agreement. Correlations between PRL-3, previous explored markers and clinicopathological variables were assessed by the Spearman Correlation test. The log-rank test was used for testing statistical significance of difference between survival curves. Survival curves were drawn by use of the Kaplan-Meier method. The curves were terminated when less than 10% of patients were still at risk (192 months). For the multivariate analyses, we used a backward Cox regression model with a probability at 0.10 for entry and 0.05 for removal. Clinicopathological variables from the univariate analyses with a $p < 0.10$ were entered. The significance level defined for all analyses was $p < 0.05$.

Ethics

This study was approved by the regional ethics committee, REK Nord, project application 2009/1393 (including a mandatory re-application which was formally approved 22.01.2016. The committee waived the need for patient consent for this retrospective study. The reporting of clinicopathological variables, survival data and biomarker expressions is in accordance with the REMARK guidelines.

Results

Expression of PRL-3

There was specific and variable cytoplasmic staining, which when present, was frequently accompanied by a granular accentuation. There was also strong nuclear staining in a subset of tumor cells. In stroma, a small subset of fibroblasts had some nuclear staining. Most lymphocytes, when present, also had a strong nuclear staining. Expression of PRL-3 was also present in benign epithelium in this study, although its extent was not systematically evaluated. Inter-observer scoring agreement was; Intraclass correlation coefficient (ICC) = 0.89 for tumor cytoplasm intensity and ICC = 0.93 for tumor cell nuclear staining. The fibroblast staining was hard to score due to very low intensity, resulting in an ICC of 0.44. Photomicrographs of low versus high expression examples of cytoplasmic tumor cell expression of PRL-3 are presented in Fig 1. For examples of IHC staining in whole tissue sections, see S1 Fig.

Of the total cohort, 397 patients had cores with morphologically verified malignant cells available for scoring. In tumor cell cytoplasm, the mean expression score was 1,25, (range 0–3) and the most prevalent score was 1 (n = 112). For tumor nuclear staining, the mean expression score was 0.48 with 0 as the most prevalent score (n = 225). For fibroblasts, only 56 had some

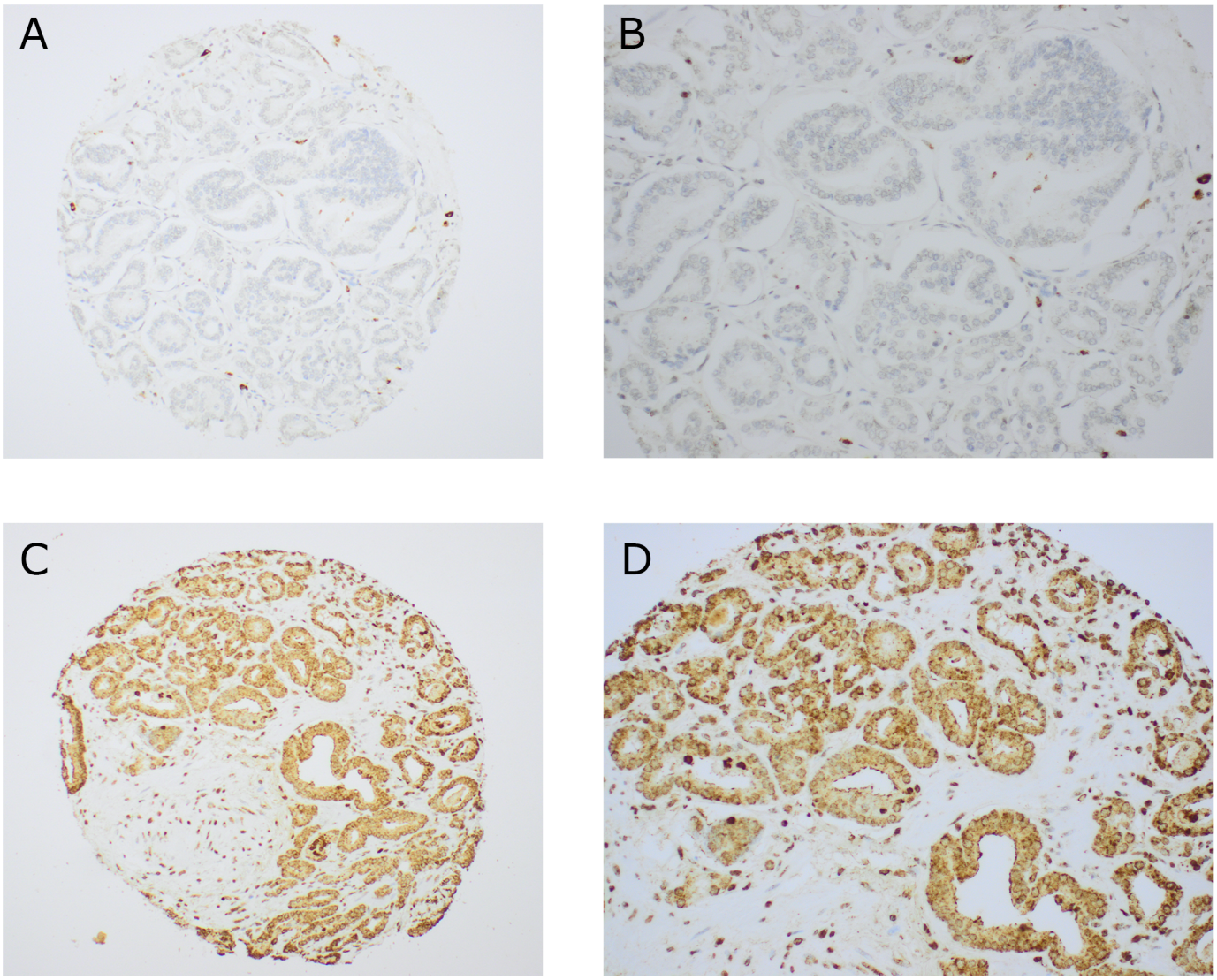

Fig 1. Illustrative examples of immunohistochemical staining for PRL3. A) a whole core at 200 magnification exhibiting low expression, B) An image of the same core as A at 400X magnification, C) a whole core at 200x showing high expression of PRL-3, D) an image taken 400x in the same core as C. This image also serves as an example of high expression in fibroblast nuclei.

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cytoplasmic staining detected by at least one of the observers. The ICC for fibroblast scoring was weak and considered unreliable for further analyses.

Correlations

We observed a positive and significant correlation between cytoplasmic and nuclear PRL-3 staining ($r = 0.42$, $p < 0.001$). There was no significant correlation with $r > 0.1$ between clinicopathological variables and cytoplasmic or nuclear PRL-3 staining. However, we found cytoplasmic PRL-3 staining to correlate to the following molecular markers previously evaluated in our cohort; tumoral VEGF-A ($r = -0.21$, $p < 0.001$), tumoral VEGFR-2 ($r = 0.22$, $p < 0.001$) and tumoral VEGFR-3 ($r = 0.31$, $p < 0.001$). The markers it did not correlate to were monocarboxylate trasporter 1 and 4, CD3, CD4, CD8, CD 20, CD56, CD68, CD138,

PD1, progesterone receptor, estrogen receptor and aromatase. For nuclear PRL-3 staining, we found no significant correlations with $r > 0.1$.

Univariate analyses

For nuclear PRL-3 expression there was no significant association to BFFS or CFFS. For cytoplasmic expression of PRL-3 we found associations between high expression of PRL-3 and poor BFFS (p = 0.131, Table 1 and Fig 2A), poor CFFS (p = 0.044, Table 1 and Fig 2B) and poor PCDFS (p = 0.041, Table 1 and Fig 2C). When exploring different cut-offs, we found a trend for worse survival for all cut-offs with variable p-values. The same tendency or significance was observed within each relevant clinicopathological subgroup (PSA, age, Gleason, pTstage, Tumor size, perineural infiltration and vascular infiltration).

Multivariate analyses

For the multivariate analyses we entered all clinicopathological variables with a p <0.10 from the univariate analyses in addition to the prognostically significant PRL-3 variable, cytoplasmic tumor cell expression of PRL-3. These variables are in bold in Table 1 and were entered in the three models according to different survival end points; BFFS, CFFS and PCDFS. However, for the last model with PCDFS there were only 18 events, which according to stringent statistical procedures, do not allow more than three variables to be entered. In all models (Table 2) cytoplasmatic PRL-3 expression in tumor cells was an independent prognosticator for poor event-free survival (BFFS, HR = 1.53, CI95% 1.10–2.13, p = 0.012; CFFS, HR = 2.41, CI95% 1.17–4.98, p = 0.017; PCDFS, HR = 3.99, CI95% 1.21–13.1, p = 0.023).

Discussion

In our large retrospective PC cohort, we found high cytoplasmic tumor cell expression of PRL-3 to be independently associated to all investigated endpoints; BF, CF and pPCdeath.

This is the first study to evaluate the prognostic impact of PRL-3 in PC. It follows a functional study on the role of PRL-3 in PC [22] and thereby further verifies its significance in PC. In addition to a functional study-based hypothesis, strengths of this study are the large well-defined cohort with long follow-up, a validated antibody using a well-adopted method (IHC), and consistent results across several endpoints. Weaknesses are the retrospective design and the lack of a training set to determine cut-offs for validation.

The many previous studies with different methods for PRL-3 detection have implicated its role in cancer, mostly demonstrating associations between high expression and poor prognosis. Associations between high protein expression and poor prognosis have been found in a variety of cancers; breast cancer[10, 26–29], colorectal cancer[7, 9, 30], gastric cancer[13, 31–34], hepatocellular carcinoma[35], cholangiocarcinoma[36], nasopharyngeal carcinoma[37], ovarian cancer[38] and adenoid cystic carcinoma [16], although there have been negative studies too [39].

Studies points to an important role of PRL-3 in cancer progression and metastasis. Initially PRL-3 was proposed as a phosphatase for metastasis[40], but multiple pathways and mechanisms have been implied to exert the effects of high PRL-3 expression. PRL-3 is a member of the PRL protein tyrosine phosphatase family and is the most studied of these so far [6, 41]. All (PRL-1, PRL-2 and PRL-3) promote proliferation, migration, invasion and metastasis[6]. PRL-3 has specifically been implicated in activation of acknowledged cancer progression pathways like phosphatidylinositol-3 kinase[42], regulating mTOR activation[43], Src tyrosine protein kinase[44, 45], epidermal growth factor receptor (EGFR)[46], and ERK[15]. Regulation of PRL-3 is found at several levels (transcriptional, translational and post translational) and is

Table 1. Patient characteristics and clinicopathological variables, and their prognostic value for biochemical failure, clinical failure and prostate cancer death in 535 prostate cancer patients (univariate analyses; log rank test).

Characteristic	Patients (n)	Patients(%)	BF (200 events)		CF (56 events)		PCD(18 events)	
			5-year EFS (%)	p	10-year EFS (%)	p	10-year EFS (%)	p
Age				0.237		**0.038**		0.404
≤ 65 years	357	67	77		94		98	
> 65 years	178	33	70		91		98	
pT-stage				**<0.001**		**<0.001**		**<0.001**
pT2	374	70	83		97		99	
pT3a	114	21	61		87		98	
pT3b	47	9	43		74		91	
Preop PSA				**<0.001**		**0.029**		**0.003**
PSA<10	308	57	81		95		99	
PSA>10	221	42	68		89		97	
Missing	6	1	-		-			
Gleason				**<0.001**		**<0.001**		**<0.001**
3+3	183	34	83		98		99	
3+4	219	41	77		94		99	
4+3	81	15	70		90		96	
4+4	17	4	58		86		94	
≥9	35	6	37		65		90	
Tumor Size				**<0.001**		**0.002**		0.085
0–20 mm	250	47	83		96		99	
>20 mm	285	53	68		90		97	
PNI				**<0.001**		**<0.001**		**<0.001**
No	401	75	80		96		99	
Yes	134	25	60		83		95	
PSM				**0.049**		0.198		0.843
No	249	47	81		96		98	
Yes	286	53	69		90		98	
Non-apical PSM				**<0.001**		**<0.001**		**0.022**
No	381	71	82		96		99	
Yes	154	29	57		85		96	
Apical PSM				0.063		0.427		0.128
No	325	61	74		92		98	
Yes	210	39	77		93		99	
LVI				**<0.001**		**<0.001**		**<0.001**
No	492	92	77		95		99	
Yes	43	8	47		69		90	
PRL-3 expression in tumor cytoplasm				0.131		**0.044**		**0.041**
Low expression	236	44	76		95		99	
High expression	161	30	72		92		96	
Missing	138	26	-		-		-	
PRL-3 expression in tumor nucleus				0.123		0.819		0.491
Low expression	225	42	71		94		98	
High expression	172	32	79		94		98	
Missing	138	26	-		-			

Abbreviations: BF = biochemical failure; CF = Clinical failure; LVI = lymphovascular infiltration; PCD = Prostate cancer death; PNI = Perineural infiltration; Preop = preoperative; PSA = Prostate specific antigen; PSM = Positive surgical margin

"Missing" corresponds to missing evaluable tumor tissue for this patient in our TMA cores.

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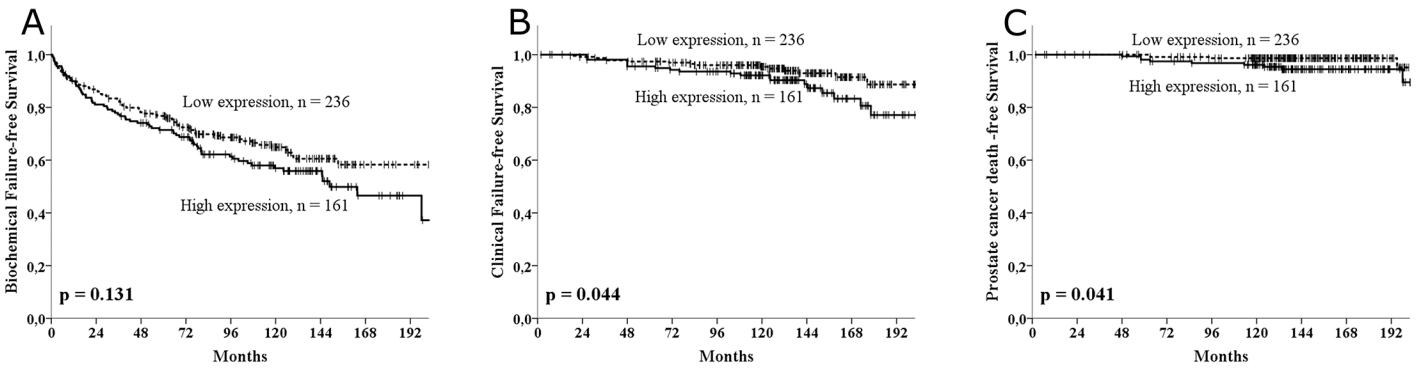

Fig 2. PRL-3 survival curves. Kaplan meier curves stratified by high and low expression of PRL-3 for A) biochemical failure-free survival, B) clinical failure-free survival and C) prostate cancer death free survival. The p-value is the univariate log rank p-value.

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mediated by several factors such as p53, TGFβ, STAT3, VEGF, Snail, PCBP1, Src etc [6, 47]. Hence, its function is complex and probably finely tuned within specific compartments.

In PC, its function has been studied in a few studies. A thorough exploration by members of our group [22] revealed several novel PC-specific findings. PRL-3 was found to be expressed at higher levels in PC tissue than in normal prostate tissue, and was ranked among the genes most differentially expressed between cancerous and benign prostate tissue. In PC cell lines, PRL-3 was present and gene amplication was found to be a possible explanation. Further, inhibition of PRL-3 hampered the PC cell lines' ability to proliferate, reduced their survival and decreased cell migration. In a small exploration of primary PC tissue and corresponding affected lymph nodes from four patients, they found no difference in expression between the metastases and primary tumor. Taken together, PRL-3 expression is probably an early event in PC tumor progression, and inhibition of PRL-3 causes reduction of pathogenic properties like migration and growth while increasing apoptosis.

This study have implications for future biomarker research in PC. In contrast to many other biomarker studies in PC, PRL-3 was significant for all clinically relevant endpoints, and it should have priority for further validation. In particular since previous biomarker studies in PC only have significant results for BF. In addition, PRL-3 has consistently been found associated with poor prognosis also in several other malignancies. Besides, PRL-3 may have potential as a therapeutic target. The findings from functional studies in various cancers including PC indicates PRL-3 to be an attractive target.

There are currently no ongoing clinical studies targeting PRL-3. However, over the last decade multiple novel PRL-3 inhibitors have been developed[48–53] and several natural occurring compounds are found to have PRL-3-inhibitory properties[54–58], both with clear *in vitro* effects on various types of cancer cells. *In vitro* studies have also investigated effects of PRL-3 inhibition on PC cells. In an explorative study on the effect of curcumin, this agent decreased PRL-3 mRNA levels in PC3 cells[59]. A marine macrolide (halichondramide) had anti-metastatic activity in highly metastatic PC3 human PC cells due to PRL-3 inhibition. The first chimeric antibody targeting PRL-3 was engineered in 2012[60]. Recently, a humanized antibody against PRL-3 (PRL3-zumab) was generated and proved effective towards human gastric cancer cells[20]. Interestingly, effects were associated with PRL-3 positive cells, suggesting expression of PRL-3 to be a possible predictive biomarker for future PRL-3 directed therapy. Our findings of RPL-3 to be primarily expressed in neoplastic and not stromal PC cells support the idea of specific tumor effects by inhibition. Though, this remains to be tested in preclinical studies prior to early phase clinical studies.

Table 2. Multivariate analyses of factors with a p < 0.10 in univariate analyses (see Table 1) for all patients (Cox regression, backward conditional). Significant p-values in bold (threshold p ≤ 0.05).

Characteristic	BF (200 events)			CF (56 events)			PCD (18 events)*		
	HR	CI95%	p	HR	CI95%	p	HR	CI95%	p
Age	NE			NS			NE		
pT-stage			**<0.001**	NS			NE		
pT2	1								
pT3a	1.56	1.04–2.33	0.031						
pT3b	3.14	1.45–3.97	**<0.001**						
Tumor Size	NS			NS			NE		
0–20 mm									
>20 mm									
Preop PSA				NS					**0.018**
PSA<10	1						1		
PSA>10	1.49	1.07–2.11	**0.02**				4.74	1.30–17.3	
ISUP grade (Gleason)	NS					**0.003**	NS		
1 (3+3)				1					
2 (3+4)				2.74	0.75–10.1	0.127			
3 (4+3)				5.39	1.40–20.7	**0.014**			
4 (4+4)				10.7	2.11–54.4	**0.004**			
5 (≥9)				10.3	2.59–41.3	**0.001**			
PNI			**0.003**	NS					**0.002**
No	1						1		
Yes	1.74	1.21–2.49					5.95	1.94–18.3	
LVI	NS					**0.007**	NE		
No				1					
Yes				3.35	1.38–8.13				
Non-apical PSM			**0.019**	NS			NE		
No	1								
Yes	1.53	1.07–2.19							
PRL-3 expression in tumor cytoplasm			**0.012**			**0.017**			**0.023**
Low expression	1			1			1		
High expression	1.53	1.10–2.13		2.41	1.17–4.98		3.99	1.21–13.1	

Abbreviations; BF = biochemical failure; CF = Clinical failure; LVI = lymphovascular infiltration; NE = not entered, due to non-significance in the univariate analyses; NS = not significant, the characteristic is removed by the backward conditional analysis due to insignificance; PCD = Prostate cancer death; PNI = Perineural infiltration; Preop = preoperative; PRL-3 = Phosphatase related to the liver- 3; PSA = Prostate specific antigen; PSM = Positive surgical margin

*Due to the low number of events in the PCD model only three variables could be entered in the model. We therefore did a careful analysis to select only the two variables other than PRL-3 that where truly independent by performing initial multiple enter analyses.

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Conclusions

This is the first study to address the prognostic impact of PRL-3 in PC. We have verified our hypothesis that high tumor cell expression of PRL-3 is a strong independent predictor for clinically relevant PC endpoints such as BF, CF and PC death. These results strongly suggest PRL-3 as a prognostic biomarker in PC, although further validation is needed. Based on the results from this study, PRL-3 is suggested as a potential therapeutic target due to expression mostly in cancer cells.

Supporting information

S1 Fig. Immunohistochemical expression of PRL-3 in whole section. PRL-3 staining in a whole section illustrating nuclear and cytoplasmic expression in both malignant and benign epithelium.
(JPG)

S1 File. Database file. This is the SPSS database file with scoring and survival data for the patients within this cohort.
(SAV)

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Author Contributions

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Writing – review & editing: Elin Richardsen, Mehrdad Rakaee, Helena Bertilsson, Roy Bremnes, Magne Børset, Lill-Tove Busund, Tobias Slørdahl.

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