The analysis of Serum Response Factor expression in bone and soft tissue Prostate Cancer metastases

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Abstract

Background. Castration resistant prostate cancer (CRPC) represents a challenge to treat with no effective treatment options available. We recently identified serum response factor (SRF) as a key transcription factor in an *in vitro* model of castration-resistance where we showed that SRF inhibition resulted in reduced cellular proliferation. We also demonstrated an association between SRF protein expression and CRPC in a cohort of castrate-resistant transurethral resections of the prostate (TURPS). The mechanisms regulating the growth of CRPC bone and visceral metastases have not been explored in depth due to the paucity of patient-related material available for analysis. In this study we aim to evaluate SRF protein expression in prostate cancer (PCa) metastases, which has not previously been reported.

Methods and Results. We evaluated the nuclear tissue expression profile of SRF by immunohistochemistry (IHC) in 151 metastatic sites from 42 patients who died of advanced PCa. No relationship between SRF nuclear expression and the site of metastasis was observed (P=0.824). However, a negative association between SRF nuclear expression in bone metastases and survival from [a] diagnosis with PCa (p=0.005) and [b] diagnosis with CRPC (p=0.029) was seen. These results demonstrate that SRF nuclear expression in bone metastases is associated with survival, with patients with the shortest survival showing high SRF nuclear expression and patients with the longest survival having low SRF nuclear expression.

Conclusion. Our study indicates that SRF is a key factor determining patients' survival in metastatic CRPC and therefore may represent a promising target for future therapies.

Introduction

Prostate cancer (PCa) is the second most frequently diagnosed cancer in developed countries and the third most common cause of death from cancer in men [1]. Early detection of PCa allows for curative therapies such as castration and radiation treatments. However, despite the emergence of new treatments, advanced disease represents a challenge to treat with no effective treatment options available [2]. Androgens play an important role during all phases of PCa growth through activation of the androgen receptor (AR) in epithelial and stromal cells [3]. The standard therapy for patients with advanced PCa is androgen ablation by surgical or medical castration. However, following initial remission, the majority of tumours eventually relapse, predominantly within the bone. These tumours are termed castration-resistant PCa (CRPC). More than 80% of all men who die of PCa have metastatic disease located within the bone [4], the majority of which are patients with CRPC. The mechanisms controlling CRPC growth in bone metastases are largely unknown and scantly explored by actually examining such bone metastases in patients.

The serum response factor (SRF) is a widely expressed transcription factor involved in cellular proliferation and cytoskeletal organisation as well as cellular growth, differentiation and resistance to apoptosis [5, 6]. Known SRF target genes are characterised by single or multiple copies of the serum responsive elements (SRE) which contain the consensus sequence CC $[A/T]_2A[A/T]_3GG$, generally known as CArG box [7]. SRF has been recently associated with PCa development and progression. SRF was firstly shown to play a role in PCa by Heemers and colleagues [8] who demonstrated that it is an important determinant of AR action through the transcriptional activation of four and a half LIM domain protein 2 (FHL2) which is up-regulated in PCa and associated with poor prognosis. In addition, inhibition of SRF has been shown to impact cellular proliferation in PCa cell lines by our group and others [2, 8]. SRF protein expression in PCa tissues has been investigated by Yu *et al.* [9] who performed immunohistochemistry on more than 400 PCa samples from radical prostatectomies. In this study, SRF expression correlated significantly with both extracapsular extension and Gleason score as well as with proliferation, while negatively correlating with apoptosis. SRF association with Gleason score has been confirmed by our group. In addition we have demonstrated that SRF is associated with castration-resistance in CRPC transurethral resections of the prostate (TURPS) [2]. The aim of this study was to evaluate SRF protein expression in PCa metastases.

Materials and Methods

Sample Collection/Tissue Microarray Construction

Human tissue microarrays were constructed consisting of 65 soft tissue metastases and 120 bone metastases from 42 patients with advanced PCa. Samples were obtained from patients who died of metastatic CRPC and who signed written informed consent for a rapid autopsy to be performed ideally within 2 hours of death, under the aegis of the Prostate Cancer Donor Program at the University of Washington [10]. Two replicate 1 mm cores of soft tissue metastases and bone metastases were taken from every case where available [11]. The tissue microarrays were assembled using the Beecher Instruments Tissue-Arrayer^{TM} (Beecher Instruments, Silver Spring, MD).

Immunohistochemical (IHC) Analysis

Immunohistochemical staining for SRF was performed using a microwave-induced antigen retrieval method. De-waxed sections were immersed in a citric acid buffer (0.01M, pH 6.0), placed in a 700W microwave oven at full power for 15 min. Using a standard avidin-biotin complex method (Vector Laboratories, Inc.), the sections were incubated with polyclonal rabbit (Santa Cruz Biotechnology, Inc. – 1:800 dilution) at 4ºC overnight. The colour reaction product was obtained with DAB and counterstained with Haematoxylin. Tonsil sections were used as positive controls. Prior to this study, the SRF antibody was subjected to western blot analysis using LNCaP cell lines which confirmed specificity for SRF (data not shown)[2].

Scoring of SRF Protein Expression and Statistical Analysis

Some unusable cores were found in the TMAs due to the tissue cores being missing, cancer necrosis, or insufficient cancer cells. These cores were excluded from the study. Nuclear immunoreactivity for SRF was assessed in soft tissue metastases and bone metastases by two independent observers (GOH) (EK). For the purpose of statistical analysis, immunoexpression of the protein was graded according to the following scales: 0, no staining, 1, faint but clearly detectable nuclear staining in >10 % of epithelial cells, 2, moderate nuclear staining in >10 % of epithelial cells and 3, strong nuclear staining in >10 % of epithelial cells.

The staining intensity of SRF in the nuclei of epithelial cells was then further divided into two groups: low expression (immunohistochemical score of 0 or 1) included those with negative or weak staining and high expression (immunohistochemical score of 2 or 3) included those with moderate or strong reactivity. Each individual's SRF positivity was calculated by obtaining an average score of their sites of [i] bone metastasis, [ii] soft tissue metastasis [iii] both bone and soft tissue metastasis.

Chi square tests and Fisher exact tests were performed on 2X2 contingency tables using IBM SPSS 20 for Windows® to test the association of SRF immunohistochemical score (positive (2/3) and (negative (0/1)) with CRPC metastases type (bone metastases versus soft tissue metastases).

The association between clinicopathological (survival times, preoperative serum PSA, age at diagnosis, PSA velocity and Gleason score) and immunohistochemical variables were visualised using scatterplots and measured using Spearman's correlation coefficient test. Log-rank test and Kaplan–Meier analyses were used for survival comparisons. The ability to predict survival times was explored using univariate Cox proportional hazard regression. All statistical analyses were performed using IBM SPSS 20.0 software and R statistical software, version 3.0.0.

Results

SRF expression in PCa Metastatic Tissue

To evaluate SRF expression in metastatic PCa, we scored IHC staining of metastatic sites from 42 patients who died of CRPC. Among 151 metastatic sites, 60 (39.7%) sites displayed positive nuclear SRF expression and 91 (60.3%) sites displayed negative SRF nuclear expression. The metastatic samples were then further divided into bone metastases versus soft tissue metastases. Out of a total of 94 bone metastatic sites, 38 (40.4%) sites had positive SRF nuclear expression and 56 (59.6%) sites displayed negative SRF nuclear expression and out of a total of 57 soft tissue metastatic sites, 22 (38.6%) sites had positive SRF nuclear expression and 35 (61.4%) sites displayed negative SRF nuclear expression (Figure 1). No association between SRF nuclear expression and PCa metastases was observed *(P=0.824)* (Table 1).

Heterogeneous expression of SRF was observed in individual patients

Of the 42 patients (Figure 2), 11 showed (26.19%) no SRF nuclear expression in any metastatic site, 6 (14.3%) had positive SRF nuclear expression in every metastatic site and 32 (76.2%) had at least one metastatic site with SRF nuclear expression. Out of the 6 patients showing SRF nuclear expression in all metastatic sites, only 3 had soft tissue metastases represented in this study.

It was noted that of the 42 patients, 8 patients (19%) had positive SRF nuclear expression in all metastatic bone sites, 12 patients (28.6%) did not express SRF in any bone metastatic sites and 22 patients (52.4%) had at least one bone metastatic site that expressed SRF. Similarly, 8 patients (19%) had positive SRF nuclear expression in all metastatic soft tissue sites, 16 patients (38.1%) did not express SRF in any soft tissue metastatic sites and 18 patients (42.9%) had at least one soft tissue metastatic site that expressed SRF. This data highlights the heterogeneity of SRF nuclear expression among different metastatic sites within the same patient.

SRF expression in PCa Metastatic Tissue correlates with survival

Only 38 patients were included in the survival analysis from diagnosis with CRPC, since this information was not available for 4 patients. A negative association between SRF nuclear expression in bone metastases and survival from [a] diagnosis with PCa (p=0.005) (Figure 3) and [b] diagnosis

with CRPC (p=0.029) (Figure 4) was seen. Based on this correlation, Kaplan Meier analysis was performed which confirmed SRF negative correlation with survival from [a] diagnosis with PCa (Log Rank test, p=0.020) (Figure 5) and [b] diagnosis with CRPC (Log Rank test, p=0.043) (Figure 6). Finally, a multivariate analysis was carried out to measure the ability of SRF to predict survival. This showed that SRF is a significant predictor of survival from [a] PCa diagnosis (p=0.012) (Table 2) and [b] CRPC (p=0.018) (Table 3). No association between SRF nuclear expression in soft tissue metastases and duration to death from [a] diagnosis with PCa (p=0.744) and [b] diagnosis with CRPC (p=0.292) was observed. Duration to death [a] from diagnosis with PCa (p=0.957) and [b] from diagnosis with CRPC (p=0.599) was not associated with the number of metastatic sites. This data show that SRF nuclear expression in bone metastases is negatively associated with survival from diagnosis with PCa and from diagnosis with CRPC, which are independent from the number of metastatic sites.

Discussion

The immunohistochemical evaluation of SRF in PCa metastatic clinical samples has not previously been reported. In this study, we evaluated the tissue expression profile of SRF in 151 metastatic sites from 42 patients who died of advanced PCa. These metastatic sites consisted of both bone metastases and soft tissue metastases from each patient.

Nuclear SRF protein expression was evaluated in both bone and soft tissue metastatic samples. SRF expression was heterogeneous among different metastatic sites within the same patient. Seventy six per cent of patients had a combination of positive and negative SRF expression within their metastatic sites. Overall, almost 40% (39.7%) of metastatic sites displayed positive nuclear SRF expression and 60% (60.3%) of sites displayed negative SRF nuclear expression. Virtually identical percentages of SRF positivity and negativity were observed when the metastatic samples were further divided into bone metastases and soft tissue metastases (40.4%, 38.6% had positive SRF nuclear expression and 59.6%, 61.4% had negative SRF nuclear expression in bone and soft tissue metastases, respectively). This confirmed that no bias of SRF expression in either bone or soft tissue metastases was affecting the SRF expression in overall metastatic tissue. Chi square tests performed on the contingency tables confirmed that there was no association between SRF nuclear positivity and negativity in either bone or soft tissue PCa metastases.

It has previously been reported by our group and others that SRF nuclear positivity was associated with higher Gleason score in primary PCa tissues [9] and castrate-resistant TURPs [2] suggesting that SRF may play a role in PCa progression. In addition, we recently showed an association between SRF nuclear positivity and castration-resistant TURPs, with 95% of castrate-resistant TURPs showing nuclear positivity for SRF [2]. However, this was not the case in PCa metastases to bone or soft tissue where only approximately 40% displayed SRF nuclear positivity. These conflicting expression levels of SRF in primary and metastatic tumour highlight how different microenvironments may influence the behavior of tumour cells. Tumour cells that metastasize to organs outside the primary tumour must survive the transit into the new environment, ultimately leading to different levels of expression of proteins compared to the primary tumour. Metastatic cancer cells often differ from the preceding primary cancer in diseases such as breast cancer, where properties such as receptor status can change, often leading to developed resistance to previous treatment. In addition,

chemotherapy and other treatments may alter the original pattern of protein expression, leading to changes between the original tumour and its metastases. Therefore, when metastatic cells progress from the primary tumour, biological heterogeneity is found within a single metastasis and among different metastases [12]. This may explain the unexpected disparity in the level of SRF expression observed in castrate-resistant primary tumour versus castrate-resistant metastatic tumour in our studies. Moreover, abundant literature has shown genetic and epigenetic heterogeneity at different metastatic sites, accounting for the phenotypic heterogeneity in prostate cancer metastases. Previous studies have shown a plethora of heterogeneous molecular aberrations in prostate cancer metastases including variable expression of the androgen receptor [11], interfocal heterogeneity of PTEN/MMAC1 gene alterations [13], differential extent of neuroendocrine differentiation [10], significantly higher E-cadherin expression in bone metastases compared with lymph node and soft tissue metastases [14] and different cell survival mechanisms in bone and soft tissue metastases, which rely on differential expression of survival proteins [15]. In addition, epigenetic mechanisms which are responsible for de-regulating key oncogenes and onco-suppressor genes are also involved in the heterogeneity of prostate cancer metastases, as shown by Yegnasubramanian and colleagues [16], who demonstrated that DNA hypomethylation patterns are quite heterogeneous across different metastatic sites within the same patient.

Although an association between SRF positivity and PCa metastases was not shown in the current study, a significant negative correlation between SRF expression in bone metastases and survival from [a] diagnosis with PCa and [b] diagnosis with CRPC was found. Moreover, univariate Cox proportional hazards modelling showed that only SRF is a significant predictor of survival from PCa and CRPC diagnosis. It should also be noted that only Gleason score 9 was found to be a significant predictor of survival from PCa diagnosis but not for CRPC diagnosis, suggesting that SRF is an independent predictor from Gleason score. These findings are in line with a previous study which showed an association between SRF expression in primary PCa tissues and poor outcome following radical prostatectomy [9]. This data also suggest that SRF plays a key role in CRPC metastases in regard to patients' survival, with patients with the shortest survival showing high SRF nuclear expression and patients with the longest survival presenting low SRF nuclear expression. Due to the heterogeneity of PCa, also evident in this study, several mechanisms may lead to the development of

metastases. However, those patients whose bone metastases are driven by up-regulation of SRF demonstrate a worse outcome than patients with low SRF in their bone metastases. Therefore, based on this data, we can speculate that SRF plays a key role in survival rates in CRPC patients, the mechanisms of which are currently under investigation in our laboratory. Interestingly, while SRF nuclear expression in bone metastases was correlated with survival, this was not the case for soft tissue metastases. This finding is supported by a recent study showing that bone metastases and soft tissue metastases rely on different cell survival mechanisms [15], therefore we can hypothesise that SRF is a key factor for bone tissue cancer cells' survival while playing a minor role in soft tissue ones. This observation requires further investigation to confirm whether or not SRF plays a role in patient survival in the setting of metastatic CRPC to bone and, if so, SRF may represent a potential target for future therapeutic intervention.

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Table and Figure Legends

Figure 1:

Serum response factor (SRF) protein expression assessed by immunohistochemistry on castration resistant prostate cancer metastases

A, Bone metastases showing strong nuclear SRF expression B, Bone metastases lacking SRF nuclear expression C, Soft tissue metastases (Lymph node) showing strong nuclear SRF expression D, Soft tissue metastases (Lymph node) lacking SRF nuclear expression. (X40 magnifications).

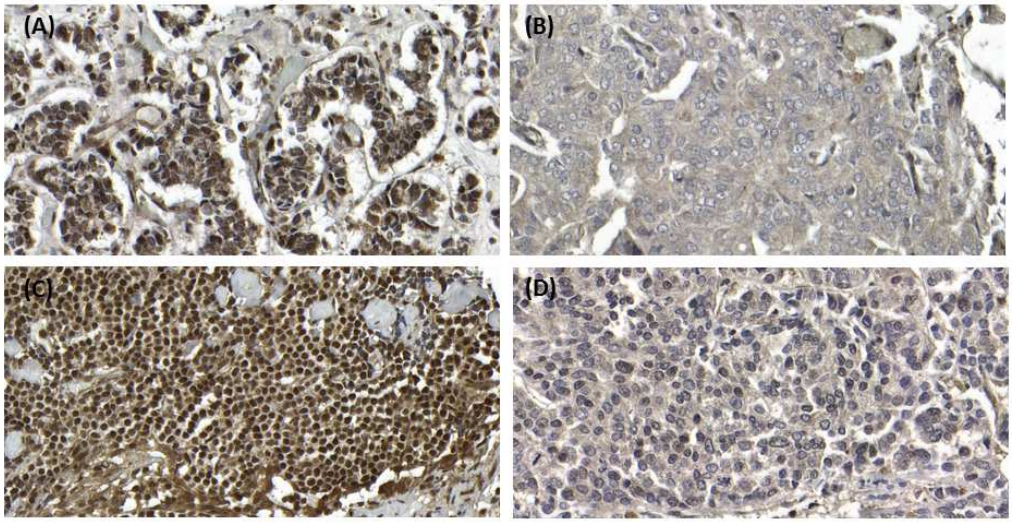

Figure 2:

The heterogeneity of SRF expression in individual patients

SRF nuclear expression performed on multiple metastatic sites of 42 PCa patients. The staining results were summarized as positive nuclear expression (dark grey) and negative nuclear expression (light grey).

Case	Soft	Soft	Bone	Bone	Bone
98-328	Peritoneal LN	Liver	Bone		
98-380	Liver		Sternum	L4	L2
98-388	Liver		Sacrum		
99-069	Peritoneal LN		R. Pelvis		L3
99-033	Lymph node		R. Iliac	T11	
99-064	R. Pelvic LN	Mesenteric LN		T9	R. Humerus
99-091	Liver	Retroaortic LN	L4	L2	R. Femur
00-010	Liver	Lymph node			
00-029	Lymph node		R. Ischium	L4	L3
00-090	Liver	Lymph node	L4	L2	T12
00-140	Lymph node	Lymph node	L2	T11	T9
00-147			4 R. Rib	R. Femur	6 R. Rib
00-169	Lymph node		L. Sacrum	L3	L2
01-046	Lung	Lymph Node	L3	T12	Sternum
01-063			L. Iliac	R. Sacrum	L3
01-087			L3	L2	T12
01-095	Liver	Lymph node	R. Iliac	L1	T9
01-181			R. Pelvis	L5	T11
02-083	Mediastinal LN	Liver	R. Sacrum	L. Sacrum	T11
02-142	Dura		L. Iliac	L4	L2
03-026	Lymph node	Liver		T9	L3
03-027	Lung	Liver	R. Iliac	L. Iliac	L4
03-077	Liver		L. Iliac		
03-081	Retroperitonial LN	Liver	L5	L2	T9
03-082	Liver				
03-130	R. Humerus				
03-139	Retroperitoneal LN	Mesenteric LN			
03-163	Liver				L1
03-192	Liver		R. Sacrum	L5	T10
04-050	Periaortic mass	Mediastinal LN	L. Sacrum	L4	T11
04-112	Iliac LN	Retroperitoneal LN			
04-149	Lymph node	Peritoneal LN	L. Sacrum	T12	T8
05-011	Periaortic LN	Liver	T12	T8	L. Humerus
05-116	Liver	Adrenal	R. Sacrum	L3	T10
05-144	Liver	Retroperitoneal LN	R. Iliac	L5	L2
05-187	Liver	Periaortic LN		Ribs	
05-214	Periaortic LN	Peravertibral LN	L5	L4	R. Humerus
05-217	Periaortic LN	Lung	L. Sacrum		
05-221			L4	T12	Ribs
06-046			L5		Ribs
06-047	R. Iliac LN	Lung	T9	R. Sacrum	
06-064			L. Iliac	L. Sacrum	L3

- SRF
+ SRF

Figure 3:

Scatterplot measuring the statistically significant correlation between SRF average bone score expression and survival from date of PCa diagnosis. (Spearman correlation coefficient = -0.423, p-value = 0.005)

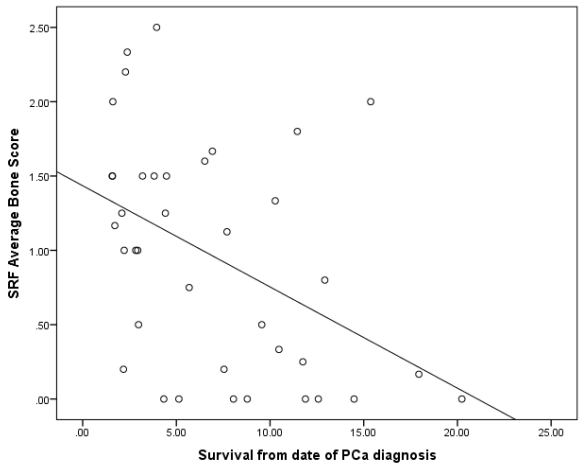

Figure 4:

Scatterplot measuring the statistically significant correlation between SRF average bone score expression and survival from date of CRPC diagnosis. (Spearman correlation coefficient = -0.334, p-value = 0.029)

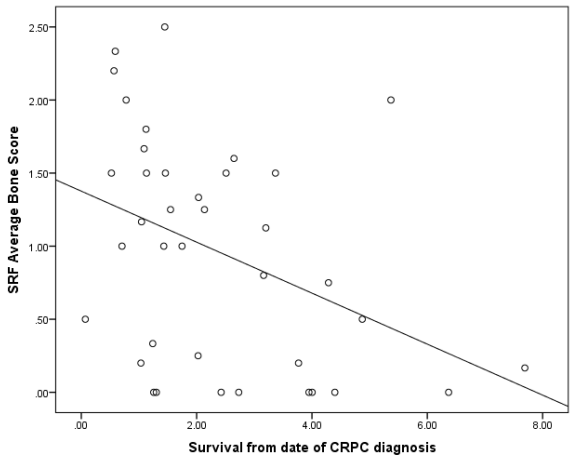

Figure 5:

Kaplan Meier cumulative survival from date of PCa diagnosis stratified by patients with low average bone score SRF expression and patients with high average bone score SRF expression (Log Rank test, p=0.020)

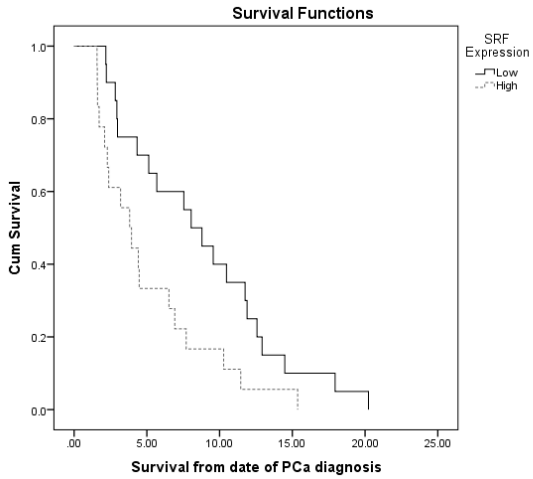

Figure 6:

Kaplan Meier cumulative survival from date of CRPC diagnosis stratified by patients with low average bone score SRF expression and patients with high average bone score SRF expression (Log Rank test, p=0.043)

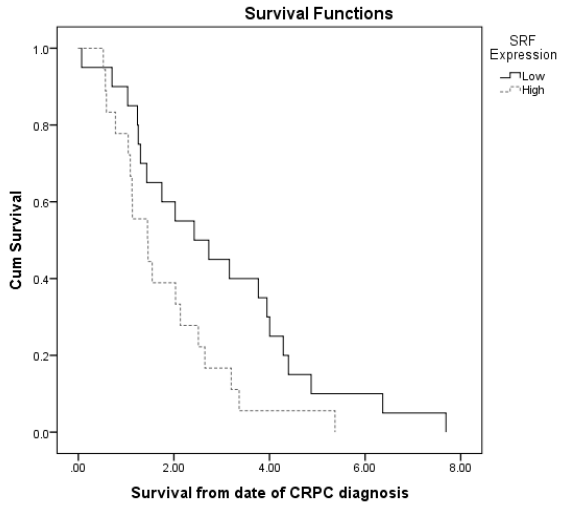

Table 1 Two-way contingency table comparing SRF expression vs. CRPC Metastases

SRF expression vs. CRPC Metastases			**SRF Score**		
			Negative	**Positive**	**Total**
Metastatic type		Bone	56 (60%)	38 (40%)	94 (100%)
		Soft Tissue	35 (61%)	22 (39%)	57 (100%)
Total			91 (60%)	60 (40%)	151 (100%)
	Value	**df**	**Asymp. Sig. 2-sided**	**Exact Sig. 2-sided**	**Exact Sig. 1-sided**
Pearson's Chi Square	.050[a]	1	0.824		
Fisher's Exact test				0.865	0.481

Table 2 Univariate Cox proportional hazard regression analysis for survival to PCa diagnosis.

	Hazard Ratio (95% CI)	***p*-value**
SRF Average bone score expression	1.682 (1.119 – 2.529)	0.012
SRF Average all sites expression	1.212 (0.786 – 1.869)	0.384
PSA Velocity	1.000 (0.999 – 1.002)	0.536
PSA at Diagnosis	1.000 (1.000 – 1.000)	0.832
Age at Diagnosis	0.959 (0.912 – 1.008)	0.100
Lead Gleason Grade		
2	Ref	0.415
3	4.665 (0.513 – 42.422)	0.172
4	2.913 (0.357 – 23.748)	0.318
5	6.152 (0.553 – 68.392)	0.139
Gleason Score		
5	Ref	0.100
6	3.862 (0.614 – 24.310)	0.150
7	1.689 (0.321 – 8.897)	0.536
8	1.663 (0.332 – 8.327)	0.536
9	5.980 (1.110 – 32.207)	0.037

Table 3 Univariate Cox proportional hazard regression analysis for survival to CRPC diagnosis.

	Hazard Ratio (95% CI)	***p*-value**
SRF Average bone score expression	1.686 (1.095 – 2.597)	0.018
SRF Average all sites expression	1.230 (0.791 – 1.914)	0.358
PSA Velocity	1.000 (0.999 – 1.001)	0.845
PSA at Diagnosis	1.000 (0.999– 1.000)	0.473
Age at Diagnosis	0.986 (0.942 – 1.032)	0.538
Lead Gleason Grade		
2	Ref	0.769
3	2.065 (0.253 – 16.844)	0.498
4	1.356 (0.170 – 10.806)	0.774
5	1.232 (0.124 – 12.200)	0.858
Gleason Score		
5	Ref	0.060
6	2.835 (0.513 – 15.968)	0.237
7	1.143 (0.224 – 5.838)	0.872
8	0.785 (0.153 – 4.030)	0.772
9	3.851 (0.789 – 18.799)	0.096

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