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Role of Serum Response Factor expression in prostate cancer biochemical recurrence

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Disclosure Statement

M Prencipe has been on secondment from University College Dublin to OncoMark from 1st September 2013 to 31st August 2014, where her official status was that of an employee; she does not hold any stock in this company. WM Gallagher is Chief Scientific Officer at OncoMark, holds stock and is a director of the company. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Abstract

Background: Up to a third of prostate cancer patients fail curative treatment strategies such as surgery and radiation therapy in the form of biochemical recurrence (BCR) which can be predictive of poor outcome. Recent clinical trials have shown that men experiencing BCR might benefit from earlier intervention post-radical prostatectomy (RP). Therefore there is an urgent need to identify earlier prognostic biomarkers which will guide clinicians in making accurate diagnosis and timely decisions on the next appropriate treatment. The objective of this study was to evaluate Serum Response Factor (SRF) protein expression following RP and to investigate its association with BCR.

Material and methods: SRF nuclear expression was evaluated by immunohistochemistry (IHC) in TMAs across three international radical prostatectomy cohorts for a total of 615 patients. Log-rank test and Kaplan–Meier analyses were used for BCR comparisons. Stepwise backwards elimination proportional hazard regression analysis was used to explore the significance of SRF

in predicting BCR in the context of other clinical pathological variables. Area under the curve (AUC) values were generated by simulating repeated random sub-samples.

Results: Analysis of the immunohistochemical staining of benign versus cancer cores showed higher expression of nuclear SRF protein expression in cancer cores compared with benign for all the 3 TMAs analysed ($p < 0.001$, $n = 615$). Kaplan-Meier curves of the 3 TMAs combined showed that patients with higher SRF nuclear expression had a shorter time to BCR compared with patients with lower SRF expression ($p < 0.001$, $n = 215$). Together with pathological T stage T3, SRF was identified as a predictor of BCR using stepwise backwards elimination proportional hazard regression analysis ($p = 0.0521$). Moreover ROC curves and AUC values showed that SRF was better than T stage in predicting BCR at year 3 and 5 following radical prostatectomy, the combination of SRF and T stage had a higher AUC value than the two taken separately.

Conclusions: SRF assessment by IHC following RP could be useful in guiding clinicians to better identify patients for appropriate follow up and timely treatment.

Key words: Biochemical recurrence, Serum Response Factor, Prostate cancer.

Introduction

Up to a third of prostate cancer patients fail curative treatment strategies such as surgery and radiation therapy in the form of biochemical recurrence (BCR) which can be predictive of poor outcome [1]. Recent clinical trials have shown that men experiencing BCR might benefit from earlier intervention post-radical prostatectomy (RP) [2-6]. Therefore there is an urgent need to identify earlier prognostic biomarkers which will guide clinicians in making accurate diagnosis and timely decisions on the next appropriate treatment.

Using a combination of transcriptomics and bioinformatics, we have identified Serum Response Factor (SRF) as an important transcription factor (TF) in an *in vitro* model of castrate-resistant prostate cancer (CRPC) [7]. SRF is a widely expressed TF involved in cellular processes relevant to cancer development and progression including cellular proliferation and cytoskeletal organization as well as cellular growth, differentiation and resistance to apoptosis [8-9]. The association of SRF protein expression with prostate cancer and its relevance to patient survival has been shown in several studies [7, 10-12]. We have demonstrated SRF clinical relevance in CRPC by immunohistochemical staining of transurethral resections of the prostate in patients who failed hormone ablation therapy with the prostate *in situ* [7]. In line with previous studies [11], we have also shown a negative association between SRF nuclear positivity in bone metastases of patients who died of prostate cancer and survival from time of diagnosis and time of castration-resistance [10]. In the same cohort of patients, we also demonstrated that higher expression of SRF correlated with shorter survival in the context of docetaxel resistance [12]. The aim of this study was to assess SRF expression in RP tissues in order to explore SRF

association with BCR with the remit of using SRF, alone or in combination with other markers, as a possible biomarker of BCR which can predict the development of distant metastases following RP.

Material and Methods

Tissue Microarrays (TMAs)

A total of 615 patients from three independent TMAs were assessed for this study. A summary of their clinical-pathological characteristics is shown in Table 1. TMA 1 was constructed from a population-based cohort of 341 prostate cancer patients who underwent open radical prostatectomy between 1998 and 2006 at the Department of Urology, Skåne University Hospital, Malmö, Sweden, using a previously described protocol [13]. From each patient, benign and malignant cores in duplicate were mounted in a total of 13 paraffin blocks. BCR was defined as a blood PSA level of at least 0.2 ng/ml with a subsequent confirmatory value. TMA 2 was constructed from a cohort of 131 prostate cancer patients who underwent open radical prostatectomy in four Irish Institutions (three tertiary referral centers and one private hospital) as part of the Irish Prostate Cancer Research Consortium (PCRC), founded in 2003. From each patient, benign and malignant cores in triplicate were mounted in a total of 14 paraffin blocks. BCR was defined, as two consecutive PSA values >0.4 ng/ml. TMA 3 included a cohort of 143 patients radically operated for prostate cancer at Ullevål hospitals in Oslo, Norway, from 1994-2001. Six cores from each patient (3 cancer+2 benign+atropy) were included in the TMA for a total of 25 paraffin blocks. BCR was defined as a blood PSA level of at least 0.1 ng/ml. Ethical approval and patient consent were previously obtained for each of the cohorts in the original sites of tissue collection.

Immunohistochemistry

Antigen retrieval of the deparaffinised tissue sections was performed using a PT-Link module (DAKO) at 95°–99°C for 20 min in a citric acid buffer (0.01M, pH 6.0). Slide staining was performed using an automated DAKO Link 48 according to the manufacturer instructions. Tonsil sections were used as positive controls for SRF staining. 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) [7].

Immunohistochemistry scoring 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 by two independent observers (MP and AF) with good agreement between the two observers. Cases for which there were discrepancies of scoring were reviewed and an agreement was found. SRF immunostaining was assessed using a nuclear score, created by multiplying each intensity level (0, no staining, 1, faint but clearly detectable staining, 2, moderate staining and 3, strong staining) by the corresponding percentage of positive epithelial cells. For the purpose of statistical analysis, the nuclear scores of SRF were then further divided into two groups: negative (immunohistochemical score <100) and positive (immunohistochemical score >100). Student T test was used to compare average SRF nuclear expression in benign vs. cancer tissues, using Microsoft Excel. Log-rank test and Kaplan–Meier analyses were used for BCR comparisons, using IBM SPSS 20.0 software. Stepwise backwards elimination proportional hazard regression analysis [14,15], using R statistical software, version 3.3.2, was used to explore the significance of SRF in predicting BCR in the context of other

clinical pathological variables. Area under the curve (AUC) values were generated by simulating repeated random sub-samples as previously published [16]. In this process the data is split 100 times randomly into training (70%) and testing data (30%). For each split, the model is fitted to the training data, and predictive accuracy is assessed using the testing data. The results are then averaged (Mean AUC values) over the splits. R statistical software, version 3.3.2, was used to generate AUC values.

Results

SRF expression in benign versus cancer tissues

SRF expression was assessed by immunohistochemistry in 3 independent TMAs made of tissues from RP from Sweden (benign/cancer tissues from 215 patients), Ireland (benign tissues from 71 patients and cancer tissues from 101 patients) and Norway (benign/cancer tissues from 131 patients) for a total of 417 benign and 447 cancer tissues from the original 615 patients. The reduction in the number of patients (30%) was due to tissue cores missing, cancer necrosis, or insufficient cancer cells, resulting in exclusion from the study. Examples of SRF staining in TMA 1 (Swedish cohort) are given in Figure 1. SRF staining was mainly nuclear with only sporadic weak staining in the cytoplasm. While SRF expression was present in stromal cells, as indicated by arrows in Figure 1, only SRF nuclear expression in the epithelial luminal cells was assessed for this study. Analysis of the immunohistochemical staining of benign cores versus cancer cores (Figure 2) showed higher expression of nuclear SRF in cancer cores compared with benign for all the 3 TMAs analysed ($p < 0.001$) (Figure 3).

SRF expression in radical prostatectomies is a predictor of BCR

In addition to exclusion of patients for technical reasons (missing tissue cores, cancer necrosis, insufficient cancer cells), patients with positive margins were also excluded from the analysis due to the fact that positive margins may be associated with BCR due to tumor cells left behind following surgery, thus confounding our analysis. We also excluded patients for whom clinical information was missing. Following these exclusions, 215 patients were available for the analysis of the correlation of SRF with time to BCR ($n=93$ for TMA 1, $n=71$ for TMA 2 and $n=51$

for TMA 3 respectively for a total of 215 patients). In order to enhance statistical power for this analysis, the 3 TMAs were combined. Examples of immunohistochemical staining are given in figure 4. Kaplan Meier analysis showed SRF negative correlation with time to BCR, with those patients with positive SRF nuclear expression, experiencing BCR earlier than those with negative SRF nuclear expression (Log Rank test, $p < 0.001$) (Figure 5). Stepwise backwards elimination proportional hazard regression analysis was carried out to explore the significance of SRF in predicting BCR in the context of other clinical pathological variables. The variables included in this analysis were: PSA at diagnosis, age at diagnosis, Gleason score, pathological T stage, country of origin and SRF expression. This statistical test involves the automated elimination of any variables that do not contribute significantly in the prediction. As shown in Table 2, after correcting for Country and T stage, SRF is still an important predictor of BCR with a hazard ratio of 1.80 and p value of 0.0521. Following on from these results, Receiver Operating Characteristic (ROC) curves were generated by simulating repeated random sub-samples. As shown in Figure 6, SRF AUC values at year 3 (AUC=0.508) and year 5 (AUC=0.566) following RP are higher than T stage AUC values (AUC=0.456 and 0.496 respectively). In addition, at any time point post-RP the full model (combination of SRF and T stage) gives higher AUC values than the single markers (AUC=0.663, 0.605 and 0.628 for year 1, 2 and 3 respectively).

Discussion

Following RP it is crucially important to monitor the risk of BCR in order to implement appropriate monitoring and timely intervene with the appropriate treatment in case of relapse. In this study SRF nuclear expression was assessed as a biomarker for BCR which may help to stratify patients to appropriate treatments in a more timely fashion. Using three independent TMAs of tissues from RP we showed that SRF expression was higher in cancer cores compared to benign cores, indicating that SRF up-regulation occurs early during prostate cancer development. These data, together with previous work by our group showing higher SRF expression in castrate-resistant vs. hormone naïve prostate tissues [7], indicate SRF as a candidate biomarker for disease progression in prostate cancer. Moreover, Kaplan-Meier curves showed SRF ability to predict BCR, with those patients with positive SRF nuclear expression, experiencing BCR earlier than those with negative SRF nuclear expression. To expand this analysis, stepwise backwards elimination proportional hazard regression analysis was carried out to explore the significance of SRF in predicting BCR in the context of other clinical pathological variables including PSA, age, Gleason score, T stage and Country of origin. The fact that the Irish cohort was significantly different from the other two could be explained by the more stringent definition of BCR for this cohort (PSA rise of at least >0.4 ng/ml vs. only 0.2 ng/ml in the Swedish and 0.1 ng/ml in the Norwegian). After correcting for Country and T stage, SRF was still an important predictor of BCR with a hazard ratio of 1.80 and a p value of 0.0521. In other words, if two men have the same T stage and are from the same Country, the man with higher SRF is 1.8 times more likely to develop BCR. Following on from these results, ROC curves and AUC values were generated at year 1, 3 and 5 post-RP. These curves show that,

while T stage is better than SRF expression in predicting BCR at year 1, SRF outperforms T stage in year 3 and 5. More importantly, the full model (combination of T stage and SRF) gives higher AUC values at each time point. These results show the high potential of SRF as a prognostic biomarker, especially for longer time points after RP when T stage loses its prognostic power.

Recently two studies have identified gene-signatures associated with high risk of relapse and metastatic prostate cancer [17, 18]. These studies used RNA platforms such as RNA-Seq [17] and gene chip microarrays [18] following RNA isolation from formalin-fixed paraffin-embedded (FFPE) material. Interestingly, some of the genes identified by these studies (LASP1, TNFRSF19, NFIB, IGFBP3, IFT57, FDPS, COL3A1, BTG2) were previously found to be de-regulated by our group, in a gene chip study comparing LNCaP cells with their castrate-resistant subline LNCaP Abl [6]. Among these genes, three are predicted SRF transcriptional targets, namely LASP1 and NFIB included in the 22 markers of the genomic classifier discovered by Erho and colleagues [18] and COL3A1, part of the 24 biomarker panel discovered by Long and colleagues [17]. This strengthens the value of using TFs such as SRF as biomarkers, due to their ability to activate/repress many downstream genes which are then found de-regulated in more complex gene-signatures. In other words, by using immune-assays such as IHC to look at the protein expression of a single TF we can gain information on its many downstream targets. In addition, IHC offers a technical advantage over RNA based platforms. While the analysis of gene-signatures combined with specific algorithms represents a powerful tool with clear cut-off values which can be easily incorporated into a commercial test, the careful morphological examination of tissues coupled with tissue microdissection to exclude stroma and benign tissues makes the process less straight forward than IHC which has the advantage of preserving

the tissue architectural structure. In the particular case of SRF, the fact that this protein is a TF represents an advantage in the scoring process since the nuclear expression is also indicative of its activity with added value to the final analysis. In addition, many IHC tests are already used routinely in the clinic (Ki67, AMCAR, PTEN etc.) making it easier to incorporate additional IHC tests in the routine workflow.

Conclusion

While further validation of SRF as a biomarker for BCR and high-risk prostate cancer is needed, our study has highlighted the importance of SRF as a marker of disease progression in prostate cancer and its possible use as a biomarker to better stratify patients for appropriate and timely treatments following RP.

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Figure and Table legends

Figure 1. SRF protein expression assessed by IHC on clinical tissues from patients with prostate cancer. Examples of staining from TMA 1 (Sweden) are shown: negative (0), weak (1), moderate (2) and strong (3) nuclear staining. Arrows indicate positive SRF staining in stromal cells (20X magnifications).

Figure 2. SRF protein expression assessed by IHC on clinical tissues from patients with prostate cancer. Representative images (TMA 1, Sweden) from benign and cancer tissues are shown at 5 X and 20 X magnification.

Figure 3. SRF protein expression in benign vs. cancer tissues. Columns represent average SRF nuclear score. Bar, standard deviations. Averages were compared using t test assuming equal variance.

Figure 4. SRF protein expression assessed by IHC on clinical tissues from patients with prostate cancer. Representative images (TMA 1, Sweden) from tissue of patient with no biochemical

recurrence (non-BCR) and tissue from patient with BCR are shown at 5 X and 20 X magnification.

Figure 5. Kaplan Meier cumulative survival from date of BCR stratified by patients with negative SRF expression (0) and patients with positive SRF expression (1) (total patient number=215).

Figure 6. ROC curves and AUC values calculated for year 1, year 2 and year 3 following RP.

Table 1. Patients' characteristics. Age and PSA average $\pm$ SD at diagnosis. GGS, Gleason score.

Table 2. Stepwise backwards elimination proportional hazard regression analysis.

Negative=0

Weak=1

Benign tissue

Cancer tissue

(Sweden)

TMA 2
(Ireland)

TMA 3
(Norway)

Non-BCR tissue

BCR tissue

Table 1. Patients' characteristics. Age and PSA average $\pm$ SD at diagnosis. GGS, Gleason score.

	Age	PSA	GGS, n patients (% of total)					T Stage, n patients (% of total)		
			≤ 5	6	7	8	≥ 9	T2	T3	T4
TMA1 Sweden	62.9 ± 5.8	8.9 ± 5.2	34 (10.4%)	109 (33.2%)	160 (48.8%)	4 (1.2%)	21 (6.4%)	165 (50.9%)	157 (48.5%)	2 (0.6%)
TMA2 Ireland	61.2 ± 6.5	8.3 ± 3.5	0 (0%)	35 (26.9%)	63 (48.5%)	21 (16.2%)	11 (8.5%)	68 (52.3%)	62 (47.7%)	0 (0%)
TMA3 Norway	60.5 ± 5.1	10.4 ± 6.7	25 (28.4%)	33 (37.5%)	24 (27.3%)	6 (6.8%)	0 (0%)	94 (74.6%)	29 (23%)	3 (2.4%)

TABLE 2. Stepwise backwards elimination proportional hazard regression analysis to BCR Diagnosis		
	Hazard Ratio (95%CI)	p-value
SRF	1.80(0.995-3.256)	0.0521
T Stage		
T2	Ref	
T3	1.88(1.055-3.337)	0.0323
Country		
Sweden	Ref	
Ireland	2.29(1.219-4.295)	0.0100
Norway	1.41(0.598-3.326)	0.4323