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Original Research Article

Therapeutic and clinico-biological significance of CREB3L4 expression in primary prostate cancer

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Abstract

Purpose: *To investigate the therapeutic, clinicopathological and biological relevancy of CREB3L4 expression in primary prostate cancer (PCa) and to determine the mechanisms underlying the deregulation of CREB3L4 expression in PCa.*
Methods: *The therapeutic, clinicopathological and biological significance of CREB3L4 expressions in two cohorts of PCa, and the mechanisms of deregulation of CREB3L4 expression using TCGA data were determined using integrative computational analyses of the clinico-genomic data of the cancer genome atlas (TCGA) and Deutsches Krebsforschungszentrum (DFKZ).*
Result: *Gene set enrichment analyses (GSEA) demonstrated enrichment of gene sets that predict biological responses to a range of approved inhibitors in the PCa subsets with low CREB3L4 expression, and at nominal and false discovery rates of $p < 0.05$ and $p < 0.25$, respectively. In addition, lower CREB3L4 expression in TCGA PCa cohort showed poorer outcomes following androgen deprivation therapy. Furthermore, GSEA demonstrated that cell proliferation, epithelial-mesenchymal transition, angiogenesis, inflammatory response and apoptosis gene sets were enriched in PCa subsets with low CREB3L4 expressions. Low CREB3L4 expression was associated with adverse clinicopathological features of PCa at adjusted $p < 0.05$. Multiple regression analysis of the methylation, microRNA expression and copy number data of CREB3L4 identified the methylation loci and miRNA expression which independently predicted the expression of CREB3L4 in PCa.*
Conclusion: *This study demonstrates the potential therapeutic relevance and clinico-biological significance of CREB3L4 expression in primary PCa.*

Keywords: *Prostate cancer, CREB3L4 expression, Gene Set Enrichment Analysis, Drug Signature Database (DSigDB), tumor biology*

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INTRODUCTION

Prostate cancer (PCa) remains one of the most important public health problems worldwide, despite the extensive efforts that has gone into elucidating the molecular mechanisms of this carcinogenesis, as well as the advancements in diagnosis and therapy that have accrued from such studies [1]. Indeed, PCa is still the 4th most commonly diagnosed cancer worldwide and the 5th most common cause of cancer-related deaths

in men, an indication of a huge knowledge gap in its pathogenesis [1].

The transcription factor, *CREB3L4*, functions downstream in the β-adrenergic signaling pathway [2]. This pathway has been demonstrated to regulate multiple cellular processes that impact the initiation and progression of cancer [3]. Although its exact physiological roles in the β-adrenergic pathway have not yet been fully elucidated, studies on *CREB3L4* have focused on its function in unfolded protein response during endoplasmic reticulum stress [4]. Specifically, *CREB3L4* appears to upregulate the expression of the cellular programs that are involved in protein processing [5]. The *CREB3L4*-regulated genes are involved in transcriptional regulation, small molecule transport, signal transduction and energy metabolism [6]. The biological roles of *CREB3L4* in cancer have been interrogated using cell lines and limited translational studies [7]. For example, studies have demonstrated that *CREB3L4* exhibits copy number gains and transcript upregulation in lung cancer and also regulates lung cancer cell invasion and migration via modulation of the *TGFB* pathway [8]. In addition, *CREB3L4* contributes to breast cancer progression through the promotion of cell cycle, cell proliferation and apoptosis [9]. Furthermore, *CREB3L4* modulates cell proliferation in PCa [10]. During *CREB3L4*-modulated PCa cell proliferation, the expression of creb3l4 protein was upregulated in prostate adenocarcinoma and high-grade prostate intraepithelial neoplasia [11]. However, the therapeutic and clinicopathological significance of *CREB3L4* have not been comprehensively investigated in a PCa cohort, nor has the mechanism involved in *CREB3L4* deregulation been studied in detail.

The specific aim of this study was to investigate the therapeutic and biological relevance of *CREB3L4* expression in primary PCa. In addition, the specific objectives of the study were to investigate the therapeutic significance of altered *CREB3L4* expression in cohorts of PCa, interrogate the clinicopathological features of CREB3L4 in primary PCa, determine if altered *CREB3L4* expression in cancer is associated with the hallmark PCa biology and to elucidate the mechanisms underlying the deregulation of *CREB3L4* expression in PCa. The study was based on some hypotheses that *CREB3L4* expression is altered in a subset of PCa cohorts, that *CREB3L4* expression may have therapeutic relevance in PCa, that *CREB3L4* expression may have associations with the hallmark PCa biology, and that *CREB3L4* is deregulated by transcriptional, translational and copy number alteration mechanisms.

METHODS

Prostate cancer cohorts

The clinicopathological and genomic data of the cancer genome atlas (TCGA) Firehose and the Deutsches Krebsforschungszentrum (DFKZ) PCa cohorts were retrieved from the Genome Data Commons (GDC) and CBioPortal for Cancer Genomics databases [12,13]. The data were analyzed for the clinicopathological and molecular correlates of *CREB3L4* expression while the mRNA and miRNA expression data were generated with RNASeq and miRNASeq technologies, respectively, and methylation data was obtained through methylation array on the Illumina Human Methylation 450 platform. The masked copy number data was generated using the Affymetrix SNP 6.0 genotyping array.

Data handling

The clinicopathological and genomic data of interest were retrieved from the GDC and CBioPortal databases, using Linux-based scripts and codes in the Ubuntu 20.04 environment in Windows. Moreover, gene expression datasets in txt and gct formats were prepared with the Linux-based codes and scripts, according to the requirements of Molecular Signature Database (MSigDB), Gene Set Enrichment Analyses (GSEA) [14,15], and DESeq2 Gene Enrichment Analyses (https://cloud.genepattern.org/) [16]. In contrast, as shown below, the phenotype and derivative gene set files were prepared in Excel spreadsheet and converted to cls and grp files, respectively. The Drug Signature Database (DSigDB; https://dsigdb.tanlab.org/DSigDBv1.0/) [17] gene set GMT files were downloaded as txt files, converted to gmt extension using Linux command lines and stored locally in computer for GSEA. The hallmark cancer biology gene sets were directly imported online from the MSigDB collection into GSEA during analysis.

TCGA cohort comprised 500 primary PCa cases with clinicopathological (i.e. prognostic and therapy outcomes), RNASeq, chromosomal copy number segment, methylation and somatic mutation data. The following amount of data was available for this cohort: clinicopathological (393 - 497 out of 500 cases for each clinicopathological index); mRNA expression (498 out of 500 cases); chromosomal copy number segment (497 out of 500 cases); methylation (322 - 498 out of 500 cases for individual methylation locus) and microRNA expression (498 out of 500 cases)

data. The DKFZ cohort comprised 118 PCa cases with clinicopathological data (including biochemical data on post-therapy recurrence) and mRNA expression data. In addition, data was available for clinicopathological features (93 - 95 out of 118 cases with RNASeq data), and RNASeq (in all 118 cases). The *CREB3L4* expression data from both PCa cohorts were converted to normally-distributed data in SPSS, prior to utilization for statistical analyses.

Ethical clearance did not apply to this study since it involved only retrospective computational analyses of open-access data from the cancer genomics databases. However, the study was carried out in accordance with the Helsinki Declaration (2008).

Study procedure

Since the therapeutic significance of *CREB3L4* expression in cancer had not previously been demonstrated, it was necessary to first interrogate both PCa cohort expression data using GSEA and kinase inhibitors response-prediction gene sets obtained from the DSigDB [17], to identify any differential enrichment between *CREB3L4*-low and *CREB3L4*-high subsets. These gene sets comprised 28 gene sets curated for the Food and Drug Administration (FDA)-approved kinase inhibitors, wherein each gene set represented a single drug or chemical compound and the associated target genes [17]. Some of the interrogated drugs are shown in Table 1.

The GSEA was reset to include only gene sets with a minimum number of fifteen (15) genes per set. Kappa statistics was used to confirm similarities of the enriched drug gene sets between the PCa cohorts, in order to validate the enrichment results. To confirm that *CREB3L4* expression levels predicted response to multiple kinase inhibitors in the FDA-approved collection, a network analysis was performed with the GSEA Enrichment Map Visualization function, using a *p-value* cut-off of 0.005 and at a false discovery rate (FDR) of 0.1. Then, the relationship between the clinicopathological indices of PCa and *CREB3L4* expression in TCGA and DFKZ cohorts was determined using the appropriate statistical tests. With the results of the clinicopathological correlates in perspective, the biological significance of *CREB3L4* expression in PCa was investigated using GSEA.

Table 1: FDA-approved tyrosine kinases, indications and mechanisms of action

Name of kinase inhibitor	Indications	Mechanisms of action, target genes or genetic alterations
Crizotinib	Non-small cell lung cancer (NSCLC)	ALK- or ROS1-positive
Nilotinib	Chronic myeloid leukemia (CML)	BCR-ABL, CKIT, PDGF
Cabozantinib	Kidney cancer, metastatic medullary thyroid cancer, hepatocellular carcinoma (HCC)	Non-specific tyrosine kinase inhibitor (TKI)
Ponatinib	CML, acute lymphoblastic leukemia (ALL)	BCR-ABL
Sorafenib	HCC, thyroid cancer, advanced renal cell cancer (RCC)	Multi-kinase inhibitor
Bosutinib	CML	SRC, ABL
Sunitinib	RCC, gastrointestinal stromal tumor (GIST)	Multi-targeted receptor TKI
Nintedanib	NSCLC	Triple angiokinase inhibitor
Vandetanib	Unresectable/metastatic medullary thyroid cancer	Kinase inhibitor
Dasatinib	CML, ALL with positive Philadelphia chromosome (Ph+ve)	Multi-kinase inhibitor
Regorafenib	Metastatic CRC, unresectable, locally advanced or metastatic GIST	Inhibitor of multiple kinases
Axitinib	Advanced RCC post-treatment failure	Second generation TKI with anti-VEGFR1, -VEGFR-2 and VEGFR-3 activities
Lenvatinib	Metastatic thyroid cancer, advanced RCC, unresectable HCC	
Dabrafenib	Melanoma, NSCLC, thyroid cancer	MAPK pathway, BRAF V600E mutation
Vemurafenib	Erdheim-Chester disease, melanoma	BRAF V600 mutation
Pazopanib	Advanced RCC, advanced soft tissue sarcoma with prior chemotherapy	Multiple protein tyrosine kinases
Palbociclib	HER2-negative, hormone receptor-positive advanced or metastatic breast cancer	Second-generation cyclin-dependent kinase inhibitor
Ruxolitinib	Myelofibrosis, polycythemia vera in patients who are refractory to steroids or who cannot tolerate hydroxyurea	Selective inhibitors of JAK1 & JAK2
Erlotinib	NSCLC, pancreatic cancer and several other types of cancer	EGFR tyrosine kinase
Gefitinib	NSCLC	Tyrosine kinases

This was performed in TCGA cohort with hallmark tumor biology gene sets such as those for cell proliferation, apoptosis, epithelial-mesenchymal transition (EMT), angiogenesis and androgen response. Gene sets with significant gene enrichment (nominal *p-value* of 0.05 and FDR of 0.05) in TCGA cohort were validated in the DFKZ cohort using core enrichment genes derived from TCGA analysis, as recommended by MSigDB. Gene Ontology Enrichment Analysis was used to verify the pathway involvement of the enriched genes in the core gene sets [18,19].

Furthermore, the mechanisms underlying the altered *CREB3L4* expression were determined in TCGA cohort from the methylation (beta values), copy number segment, and miRNA expression data. Moreover, differential enrichment of miRNAs between *CREB3L4*-low and *CREB3L4*-high cases was assessed using the online DESeq2 software on the GenePattern computing environment. Then, the significantly enriched miRNAs were subjected to regression analysis, together with the methylation and *CREB3L4* copy number indices, to determine their roles in the deregulation of *CREB3L4* expression.

Statistical analysis

The default parameters of the GSEA and DESeq2 software were used in the gene enrichment analyses, except the correction for GSEA multiple testing which was set at an FDR of 0.05 (or 5 %). The clinicopathological and genomic data of TCGA and DFKZ PCa cohorts were input into SPSS version 29. Associations between categorical variables were defined with Chi-square (or Fisher) test, while the correlations between continuous variables were tested with bivariate correlative analysis.

The mean differences of continuous variables between discrete groups were determined with one-way ANOVA test while the predictive relationship between *CREB3L4* expression and the established mechanisms involved in altered gene expression (*CREB3L4* copy number variation, *CREB3L4* promoter methylation and *CREB3L4*-specific miRNA expression patterns) were ascertained with multiple linear regression analysis.

The prognostic significance of *CREB3L4* expression was defined using Kaplan-Meier and Cox regression analyses. A $p < 0.05$ was used as the threshold for significant tests, while the Benjamini-Hochberg correction was used to correct for multiple testing at an FDR value of < 0.05.

RESULTS

CREB3L4 expression levels predicted response to multiple kinase inhibitors in PCa

Results from drug GSEA using drug response-prediction gene sets from the DSigDB collections demonstrated that PCa cases with low *CREB3L4* expression in TCGA and DFKZ cohorts were enriched for genes that predict response to multiple kinase inhibitors in the FDA stable, at nominal $p < 0.05$ and FDR **<** 0.25. The *CREB3L4*-low PCa cases were enriched for the target genes of all the drugs shown in Table 1, except for erlotinib and gefitinib. In contrast, the *CREB3L4*-high cases did not show any enrichment in drug targets. Kappa statistics revealed that there was perfect concordance between TCGA and DFKZ PCa cohorts in drug response prediction *via CREB3L4* expression levels (percentage concordance = 100 %; kappa = 1.000; standard error of kappa = 0.000; 95 % confidence interval = 1.000). Network analyses revealed significant interactions among the gene targets for multiple kinase inhibitors in both cohorts (Figure 1), suggesting that *CREB3L4* levels are predictors of the levels of expression of gene targets of multiple drugs. Moreover, Leading Edge Analyses of the two PCa cohorts identified the most frequently enriched gene subsets among the drug-response gene set collection (Figure 1). A comparison of the top 20 leading-edge genes in either PCa cohort showed a percentage concordance of 75 % of genes, indicating evidence of a high rate of agreement between the two cohorts.

Therapy-resistance correlates of *CREB3L4* expression

The association of *CREB3L4* expression with the outcome of androgen deprivation therapy (ADT) was tested in TCGA cohort. Chi-square test showed that low *CREB3L4* expression was associated with progressive disease following treatment with ADT ($\chi^2 = 7.993$, $p = 0.005$). Next, bivariate correlation analysis was used to investigate the relationship between *CREB3L4* expression and the expressions of the ADT resistance-associated genes (*AR*), GCR and MLR. The analysis demonstrated inverse correlations between *CREB3L4*-MLR expression and *CREB3L4*-GCR expression (MLR: $R = -0.216$; $p < 0.001$; GCR: $R = -0.352$; $p < 0.001$). However, there was no significant correlation in expression between *CREB3L4* and *AR*. Then, a

binary logistic regression analysis was performed to determine if each of the genes *CREB3L4*, *AR*, MLR and GCR independently predicted the outcome of ADT in TCGA PCa cohort. The regression analysis showed that only the expression levels of *CREB3L4* and *AR* independently predicted outcome in the regression model (-2 log-likelihood = 203.064; Nagelkerke R^2 = 0.216; Hosmer and Lemeshow test: p = 0.776; Table 2). Low *CREB3L4* and high *AR* expressions predicted less-than-complete response. Although no data on therapeutic outcome was available for the DFKZ cohort, *CREB3L4* expression in the DFKZ cohort was inversely correlated with GCR (R = - 0.208; p = 0.044), but not with *AR* or MLR. Kaplan-Meier analysis revealed that *CREB3L4* expression did not correlate with the time taken for biochemical recurrence of tumor in TCGA PCa cohort (Log-Rank test, p = 0.279). However, while 7 of the 30 *CREB3L4*-high cases had a biochemical recurrence in the cohort, 11 of the 40 *CREB3L4*-low cases had recurrences. In the DFKZ cohort, Kaplan-Meier analysis of the association of *CREB3L4* expression with a time lag before biochemical recurrence did not indicate statistical significance (Log-Rank test, p = 0.134). However, 11 of the 39 *CREB3L4*-low cases had biochemical recurrence, whereas biochemical recurrence was seen only in 6 of the 42 *CREB3L4*-high cases.

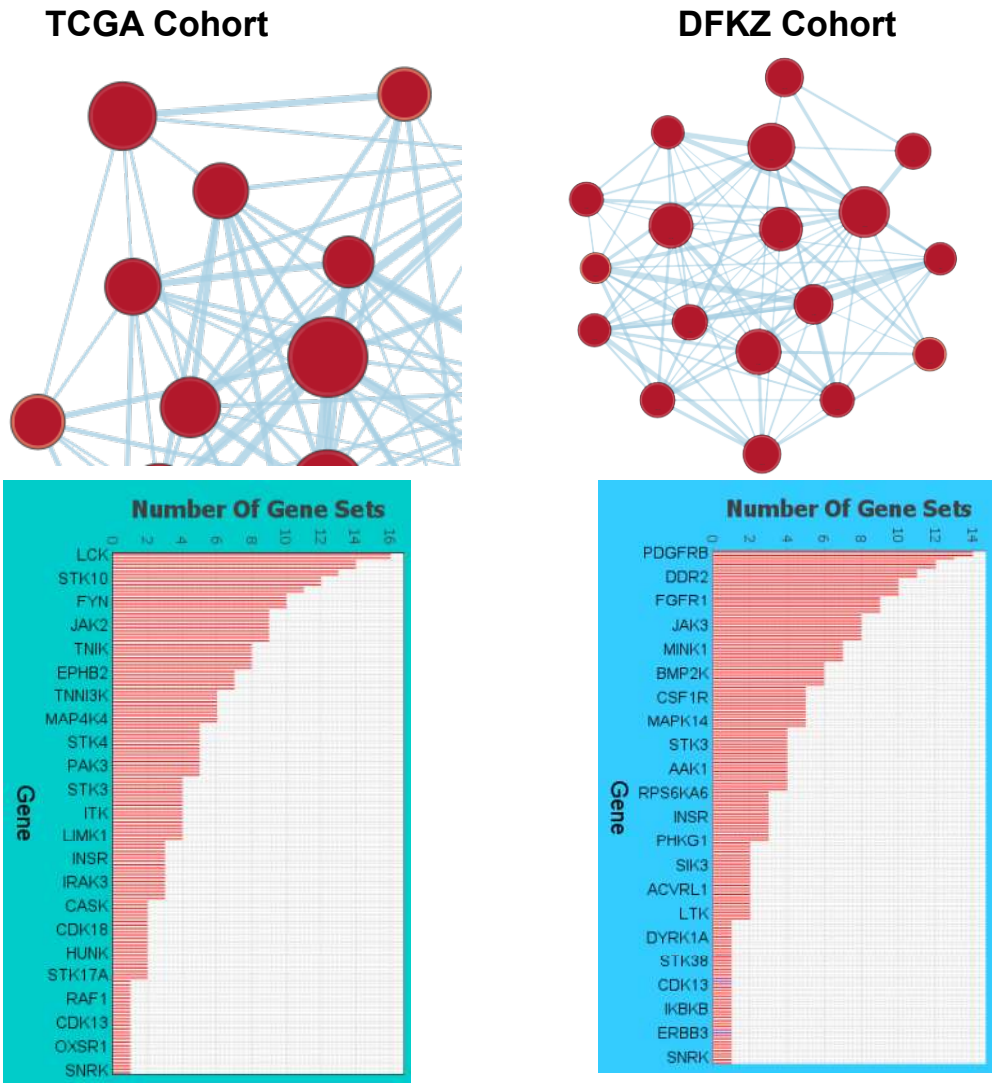

Figure 1: *CREB3L4* expression levels predicted response to multiple kinase inhibitors in PCa. Upper panel: Gene set enrichment maps showing networks of FDA-approved kinase inhibitor target gene sets in the *CREB3L4*-low subsets of TCGA and DFKZ PCa cohorts. The nodes represent the kinase inhibitor gene sets, while the edges represent overlaps in the gene sets denoting that the gene sets share common genes. The node sizes denote the sizes of the gene sets. Lower panel: Leading Edge Analysis charts showing the genes that are commonly enriched among the kinase inhibitor gene sets in TCGA and DFKZ cohorts

Table 2: Binary logistic regression analysis of therapy outcomes in TCGA PCa cohort

Regressor	B	SEM	df	*P*-value	Exp (B)
MLR expression	0.133	0.209	1	0.526	1.142
GCR expression	-0.105	0.082	1	0.201	0.900
AR expression	0.096	0.025	1	<0.001	1.101
CREB3L4 expression	1.768	0.419	1	<0.001	5.857
Constant	-4.027	0.689	1	<0.001	0.018

Low *CREB3L4* expression is associated with adverse clinicopathological features of PCa

One-way ANOVA was used to test the mean differences in *CREB3L4* expression between and among discrete categories of the clinicopathological features in the two PCa cohorts. Specifically, there were significantly lower mean *CREB3L4* expressions in adverse categories of primary Gleason pattern, secondary Gleason pattern, pathological tumour stage, pathological node stage, pathological metastasis stage, TNM stage and ISUP grade group (Table 3), and also in poorer therapy outcomes (Table 4) than in the more favourable categories in TCGA cohort. Similarly, the mean *CREB3L4* expression was down-regulated in the adverse categories of primary and secondary Gleason patterns, pathological tumour stage, overall disease stage and ISUP grade group in the DFKZ cohort (Table 5). Chi square test with dichotomised *CREB3L4* expression median values was used to confirm the findings from one-way ANOVA test (Figure 2). The results showed that low-*CREB3L4* expression was associated with the aforementioned adverse clinicopathological features of PCa in both cancer cohorts, in keeping with the characteristics of a tumour suppressor gene (TSG). However, Kaplan-Meier analysis tests revealed that there was no association between *CREB3L4* expression and overall survival (Log-Rank test: p = 0.458) or disease-free survival (Log-Rank test: p = 0.466) in TCGA cohort.

Differential enrichment of tumour-promoting biological pathways in *CREB3L4*-low PCa subsets

The *CREB3L4*-based GSEA was performed using the gene set permutation option and a signal-to-noise metric for ranking genes. The results showed enrichment of EMT, epithelial cell proliferation, apoptosis, angiogenesis, inflammatory response and transforming growth factor beta signalling gene sets in the *CREB3L4*-low class of TCGA cohort.

Table 3: Pathological correlates of *CREB3L4* expression in TGCA prostate cancer cohort

Pathological feature		n	Mean *CREB3L4* expression	Std. Deviation	*F*	Adjusted *P*-value
Gleason score	Gleason-Low	293	111.575	36.484	13.379	0.002
	Gleason-High	204	99.452	36.155		
	Total	497	106.599	36.800		
ISUP prognostic grade group	Group I	44	111.467	41.326	5.227	0.002
	Group II	147	110.043	35.910		
	Group III	101	113.866	35.509		
	Group IV	65	109.200	35.537		
	Group V	140	95.004	35.554		
	Total	497	106.599	36.800		
Pathological Tumour Stage	pT2	181	115.014	35.252	9.821	0.002
	pT3	269	100.317	35.541		
	pT4	10	92.505	52.063		
	Total	460	105.930	36.509		
Lymph node status	Negative	321	107.389	36.867	8.750	0.005
	Positive	76	93.776	32.477		
	Total	397	104.783	36.426		
Metastasis status	Negative	455	106.498	36.019	6.830	0.013
	Positive	3	52.026	26.993		
	Total	458	106.141	36.213		
Overall stage (TNM)	Localized	135	114.257	36.010	14.630	0.002
	Advanced	259	99.755	35.561		
	Total	394	104.724	36.330		

Table 4: Correlates of *CREB3L4* expression in TGCA prostate cancer cohort

Feature		n	Mean *CREB3L4* Expression	Std. Deviation	*F*	Adjusted *P*-value
Age at diagnosis	≤60 years	223	105.042	33.865	0.724	0.461
	≥61 years	274	107.866	39.042		
	Total	497	106.599	36.8002		
Race/ethnicity	A/Indian	1	80.413		0.544	0.703
	Asian	12	113.310	31.558		
	Black	57	109.793	36.275		
	White	412	105.584	36.567		
	Total	482	106.222	36.374		
	pT3	269	100.317	35.541		
	pT4	10	92.505	52.063		
	Total	460	105.930	36.509		
Treatment outcomes	Complete	224	112.932	37.912	19.712	0.002
	Others	44	85.836	31.927		
	Total	268	108.483	38.285		

Table 5: Clinicopathological correlates of *CREB3L4* expression in DFKZ prostate cancer cohort

Clinicopathological feature		n	Mean *CREB3L4* Expression	Std. Deviation	*F*	Adjusted *P*-value
Age at diagnosis	32-36 years	1	128.369		0.179	0.910
	37-41 years	4	96.355	22.699		
	42-46 years	32	106.712	42.038		
	47-52 years	57	105.611	40.850		
	Total	94	105.834	40.251		
Pathological tumour stage	pT2	61	117.348	33.097	11.600	0.002
	pT3	28	93.788	40.615		
	pT4	3	30.185	26.550		
	Total	92	107.335	39.333		
Gleason score (primary pattern)	Gleason pattern 3	69	116.182	34.082	19.710	0.002
	Gleason pattern 4	18	93.895	37.847		
	Gleason pattern 5	7	34.533	17.284		
	Total	94	105.834	40.251		
ISUP grade group	Group I	13	128.498	43.107	9.860	0.002
	Group II	56	113.323	31.409		
	Group III	11	105.239	42.697		
	Group IV	1	80.705			
	Group V	13	53.347	28.309		
	Total	94	105.834	40.251		
Pre-operative PSA	Low PSA	47	112.076	37.388	1.402	0.304
	High PSA	45	102.384	41.099		
	Total	92	107.335	39.333		

The results were validated in the DFKZ cohort using the core enrichment genes obtained from TCGA analysis. A detailed examination of the core gene sets using PANTHER pathway gene ontology analysis tool showed enrichment of pathways and gene ontology terms associated with EMT, cell proliferation, angiogenesis, inflammatory response and apoptosis (Figure 3).

The results demonstrate the association of *CREB3L4* expression with tumour biology. Moreover, the results show that *CREB3L4* exhibit the characteristics of a TSG in PCa, as the loss of expression or negative expression of *CREB3L4* was associated with enrichment of genes that promote carcinogenesis.

Deregulation of *CREB3L4* expression in PCa

The deregulation of *CREB3L4* expression was investigated in TCGA cohort, as it has a comprehensive data on copy number segment, miRNA expression and methylation. The copy number status of *CREB3L4* was obtained from the masked copy number data using the segment mean thresholds of -0.3 and 0.3. Using these thresholds, there were 21 out of 495 cases with gains/amplifications and 474 out of 495 cases with copy neutral status. One-way ANOVA test showed that there was no significant difference in mean *CREB3L4* expression between cases with *CREB3L4* gain/amplification and those with *CREB3L4* copy-neutral status (p = 0.118).

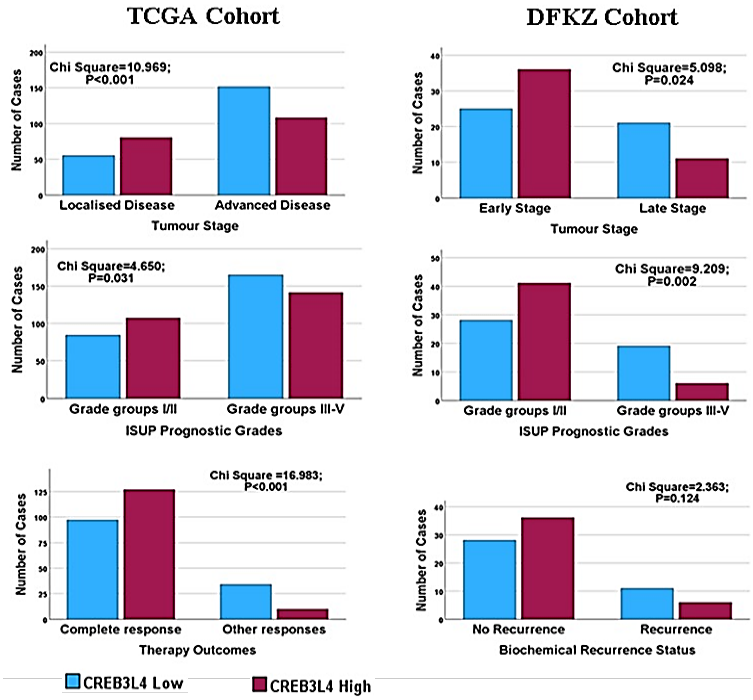

Figure 2: Clustered bar charts showing associations of *CREB3L4* expression with clinicopathological features of TCGA and DFKZ PCa cohorts

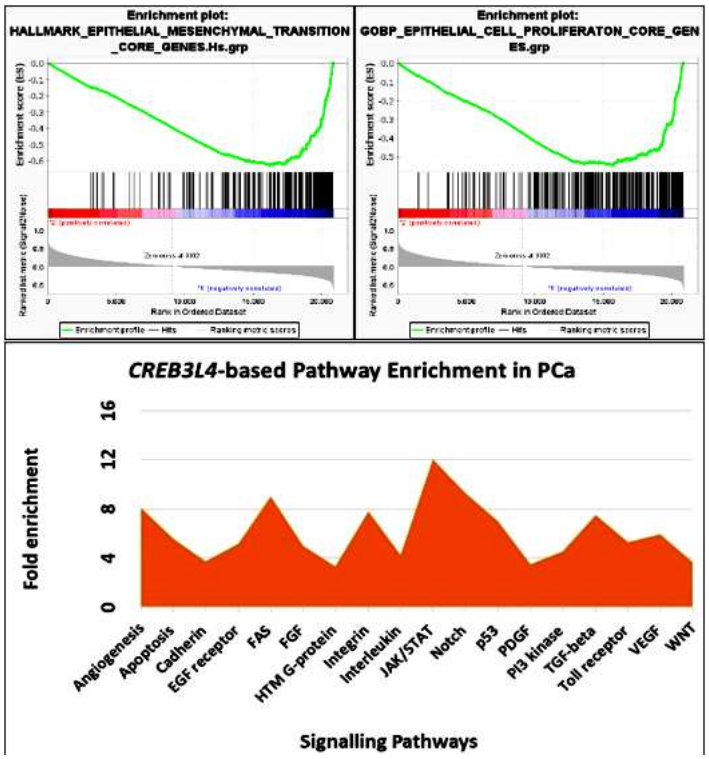

Figure 3: Differential enrichment of tumour-promoting biological pathways in *CREB3L4*-low PCa subsets. Top panel: Enrichment plots for EMT and cell proliferation, which are some of the core gene sets enriched in the *CREB3L4*-low PCa subset. Bottom panel: Overlaid area chart of differences showing gene ontology enrichment in selected core enrichment gene sets. The pathways shown here were enriched at *p*-values ≤ 0.05. The gene ontology enrichment analysis was performed on www.pantherdb.org

The top 40 differentially expressed miRNAs between *CREB3L4*-low and *CREB3L4*-high PCa subsets were identified using differential enrichment analysis DESeq2 at the default adjusted *p* value of 0.1. Bivariate correlation analysis and one-way ANOVA identified 17 out of 40 miRNAs whose expressions correlated with *CREB3L4* expression (Table 6). The beta value of 15 *CREB3L4* methylation probes were retrieved from TCGA PCa methylation data and used to interrogate the relationship between *CREB3L4* expression and methylation.

A combination of bivariate correlation analysis and one-way ANOVA identified 8 methylation probes that showed correlations with *CREB3L4* expression, out of a total of 15 methylation probes. These were cg07556888, cg09895920, cg11532795, cg12464233, cg17818873, cg22228373, cg25064552 and cg09335321 (Table 7). All identified miRNA and methylation loci were incorporated into a multiple linear regression to test whether they independently predicted *CREB3L4* expression in PCa. This analysis resulted in the identification of hsa-mir-452 ($p < 0.0\ 01$), hsa-mir-330 ($p < 0.001$), hsa-mir-30a ($p < 0.001$), hsa-mir-24-2 ($p < 0.001$), cg09895920 ($p < 0.001$), hsa-mir-150 ($p = 0.004$), cg25064552 ($p = 0.006$), hsa-mir-7641-2 ($p = 0.004$), cg17841099 ($p = 0.026$) and hsa-mir-7156 ($p = 0.041$) as independent predictors of *CREB3L4* expression in the regression model ($F = 28.199$, adjusted $R^2 = 0.370$; $p < 0.001$; Figure 4). In this study, the copy number alteration status of *CREB3L4* did not predict *CREB3L4* expression. Overall, the results showed that *CREB3L4* expression in PCa was deregulated mainly by miRNA and epigenetic mechanisms.

DISCUSSION

This study has demonstrated that low *CREB3L4* expression is associated with over-expressions of gene targets for multiple FDA-approved kinase inhibitors, which are used for the treatment of several cancer types [20]. The demonstration of enrichment of gene targets of multiple type of kinase inhibitors in *CREB3L4*-low primary PCa cases has some interesting implications.

Table 6: Correlation of microRNA and *CREB3L4* expression

miRNA species	Number of cases	Pearson's correlation	*P*-value
hsa-mir-1247	492	-0.248	<0.001
hsa-mir-1269a	492	0.024	0.594
hsa-mir-1304	492	0.044	0.327
hsa-mir-138-1	492	-0.051	0.262
hsa-mir-138-2	492	-.090*	0.045
hsa-mir-146a	492	-0.188	<0.001
hsa-mir-150	492	-0.176	<0.001
hsa-mir-181b-2	492	-0.165	<0.001
hsa-mir-195	492	-0.212	<0.001
hsa-mir-2114	492	-0.005	0.911
hsa-mir-24-1	492	-0.407	<0.001
hsa-mir-24-2	492	-0.410	<0.001
hsa-mir-26a-1	492	-0.325	<0.001
hsa-mir-26a-2	492	-0.326	<0.001
hsa-mir-30a	492	0.270	<0.001
hsa-mir-330	492	-0.330	<0.001
hsa-mir-3652	492	-0.018	0.684
hsa-mir-452	492	-0.408	<0.001
hsa-mir-4521	492	0.093	0.040
hsa-mir-4522	492	0.022	0.627
hsa-mir-4523	492	-0.058	0.202
hsa-mir-4524a	492	-0.001	0.984
hsa-mir-4525	492	-0.005	0.913
hsa-mir-4526	492	-0.049	0.276
hsa-mir-4527	492	0.030	0.507
hsa-mir-4528	492	-0.073	0.106
hsa-mir-4529	492	-0.127	0.005
hsa-mir-486-1	492	-0.039	0.385
hsa-mir-486-2	492	-0.039	0.386
hsa-mir-612	492	-0.011	0.815
hsa-mir-6125	492	0.032	0.474
hsa-mir-7156	492	-0.107	0.017
hsa-mir-7641-2	492	0.204	<0.001

Table 7: Correlation between *CREB3L4* expression and methylation status

Methylation loci	Number of cases	Pearson's correlation	*P*-value
cg00702693	496	-0.086	0.055
cg07556888	496	-0.138	0.002
cg07948599	496	0.015	0.731
cg09335321	496	-0.094	0.036
cg09867184	496	-0.069	0.126
cg09895920	496	-0.281**	<0.001
cg11224232	496	-0.062	0.171
cg11532795	496	-0.116	0.010
cg12464233	496	-0.141	0.002
cg17493511	496	-0.031	0.484
cg17818873	496	-0.149	0.001
cg17841099	496	-0.074	0.098
cg22228373	496	-0.113	0.012
cg25064552	495	-0.164	<0.001
cg26036199	496	0.016	0.717

*The dependent variable is therapy outcome (complete versus other responses#)
"Other responses" comprised partial response, stable disease and progressive disease

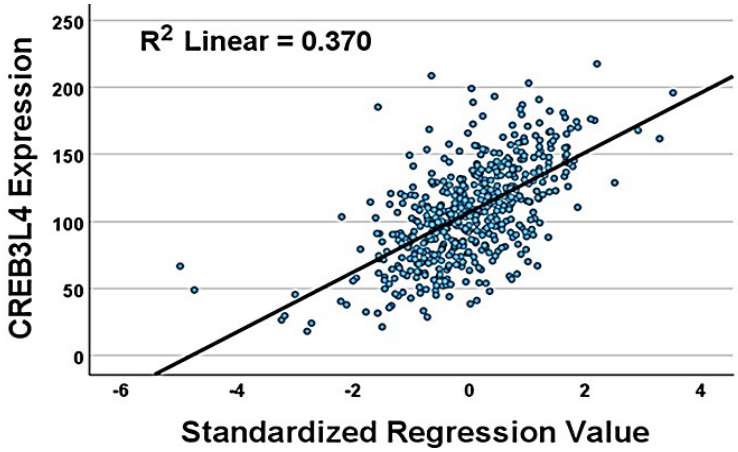

Figure 4: A scatterplot showing the correlation between *CREB3L4* expression and the multiple regressors (methylation loci and miRNA species)

The first is that a non-target of a drug may function as a predictive marker for that drug response. *CREB3L4* is not a known direct or indirect target for the group of kinase inhibitors interrogated in this study. However, by virtue of coordinate upregulation and downregulation of a non-target with the gene targets of the drugs, *CREB3L4* may assume the role of a predictive biomarker [21]. Secondly, it is noteworthy that *CREB3L4* is associated with the enrichment of gene targets of multiple kinase inhibitors with different mechanisms of action. This feature whereby a single marker could predict response to multiple therapeutic agents may engender an integrated approach to cancer therapy.

The concept of utilizing a more integrated approach with multiple biomarkers and drugs is attractive with respect to biomarker discovery and biomarker-directed chemotherapy [22]. This would increase the therapeutic options available to the oncologist and also encourage the practice of combination chemotherapy while upholding the principles and practice of precision medicine [23]. In cancer therapy, combination chemotherapy has been hailed as a veritable strategy due to its tendency to reduce toxicity in patients, and at the same time lower the risks of drug resistance through the utilization of multiple mechanisms of action [23]. Furthermore, the results bring to bear the notion that high throughput genomic methods such as RNASeq, in combination with computational analyses, would eventually find a more dominant role in biomarker discovery and biomarker-directed management of cancer and other diseases [24].

These high throughput methods have the potential to significantly improve precision medicine approaches by addressing the

problems of availability of therapeutic options for conditions that hitherto had limited or no opportunities for targeted therapy [24]. Furthermore, with regard to its potential role as a therapeutic response marker, low *CREB3L4* expression was found to be associated with poor therapeutic outcomes for TCGA patients who received androgen-deprivation therapy. However, this finding could not be replicated in the DFKZ cohort due to unavailability of treatment outcome data.

This study also demonstrated that low *CREB3L4* expression was associated with the adverse clinicopathological features of PCa, as well as the upregulation of hallmark tumour biology, in keeping with the characteristics of altered TSG in cancer. The findings from this study appear to contradict the roles that have been ascribed to *CREB3L4* in other studies [9,10,25]. These works, which are limited to mainly cell lines, described oncogenic roles for *CREB3L4* in different cancer types, which roles involve the promotion of invasion and metastasis, cell cycle, cell proliferation and inhibition of apoptosis [8-10]. However, cell line studies may not replicate all aspects of the tumour biology of any given cancer type for pertinent reasons that have been extensively reviewed by Wilding and Bodmer [26]. For these reasons, the cell line models described for *CREB3L4* oncogenic functions may be inadequate for evaluating the full biological spectrum of *CREB3L4* activities in cancer.

On the other hand, in light of its physiological roles in maintaining cellular homeostasis through unfolded protein response [5-7], *CREB3L4* may be considered a TSG. Furthermore, some genes may have both oncogene and tumour suppressor activities, a phenomenon called TSG-oncogene duality, wherein the prevailing activities of the genes at any given time depend on the molecular context in which they exist [27]. The concept of TSG-oncogene duality is not an uncommon phenomenon in cancer and it usually involves transcriptional regulators of gene expression. It has been proposed that many commonly known cancer-related genes such as *TP53, BRCA1, DNMT1, DNMT3A, ETV6, EZH2, FOXA1, FOXL2, FOXO1, FOXO3, FOXO4, KLF4, KLF5, NCOA4, NOTCH1, NOTCH2, NOTCH3, NPM1, PML, PPARG, RB1, RUNX1, SMAD4, STAT3, TCF3, TCF7L2, TP53, TP63,* and *WT,* possess TSG-oncogene duality and are also described as proto-oncogenes with tumour suppressor functions (POTSFs, or “double agents”) [28]. Incidentally, the *CREB3L4* homologue, *CREB3L1*, was also identified as one such gene [28]. The TSG-oncogene duality of *NKX2-1* (also known as *TTF1)* is described in detail in the review [27]. If the findings of this study are placed side-by-side with the data from the currently available literature on *CREB3L4*, an inference could be drawn to the effect that *CREB3L4* is a “double agent” in the regulation of cancer biology. Currently, studies on *CREB3L4* are limited to a handful of lung and prostate cancers without clinicopathological correlates [2,11]. Therefore, this study may be the first to interrogate the clinicopathological features of *CREB3L4* expression in PCa. Hence no frame of reference exists for comparison of the clinicopathological characteristics of *CREB3L4* expression in PCa. Finally, this study has demonstrated that *CREB3L4* expression is deregulated by epigenetic and miRNA mechanisms which are the common gene dysregulation mechanisms in cancer [29].

CONCLUSION

This study demonstrates that *CREB4L4* expression is associated with enrichment of targets for multiple kinase inhibitors and may therefore be a predictive biomarker of response to an integrated cancer therapy approach. The study also demonstrates that low *CREB3L4* expression is associated with adverse clinicopathological features of PCa, as well as tumour-promoting cancer biology, thereby suggesting a tumour suppressor role for *CREB3L4*. However, in the light of its demonstrated roles as an oncogene in mechanistic studies, this study hereby proposes that *CREB3L4* may exhibit the TSG-oncogene duality in cancer. Therefore, there is need for further investigations to unravel the molecular contexts of its functions.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The author declares that this work was done by the author named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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