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Associations of CTCF and FOXA1 with androgen and IGF pathways in men with localized prostate cancer.

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Short Title: PCa and CTCF/FOXA1 associations with AR/IGF signaling

Abstract

Aims: To examine associations between the transcription factors CCCTC-binding factor (CTCF) and forkhead box protein A1 (FOXA1) and the androgen receptor (AR) and their association with components of the insulin-like growth factor (IGF)-pathway in a cohort of men with localized prostate cancer.

Methods: Using prostate tissue samples collected during the Prostate cancer: Evidence of Exercise and Nutrition Trial (PrEvENT) trial (N=70 to 92, depending on section availability), we assessed the abundance of CTCF, FOXA1, AR, IGFIR, p-mTOR, PTEN and IGFBP-2 proteins using a modified version of the Allred scoring system. Validation studies were performed using large, publicly available datasets (TCGA) (N=489).

Results: We identified a strong correlation between CTCF and AR staining with benign prostate tissue. CTCF also strongly associated with the IGF-IR, with PTEN and with phospho-mTOR. FOXA1 was also correlated with staining for the IGF-IR, with IGFBP-2 and with staining for activated phosphor-mTOR. The staining for the IGF-IR was strongly correlated with the AR. **Conclusion:** Our findings emphasise the close and complex links between the endocrine controls, well known to play an important role in prostate cancer, and the transcription factors implicated by the recent genetic evidence.

Introduction

Prostate Cancer (PCa) is a leading cause of cancer-related mortality in men. Although the pathogenesis of PCa remains largely unknown, some risk factors have been recognized including increased BMI, increased consumption of dairy products, ethnicity, family history and age. Genome-wide association studies (GWAS) have identified thousands of single nucleotide polymorphisms (SNPs) within more than 160 loci that are associated with the risk for PCa; together these explain up to 28% of the familial risk for PCa [1]. A few of these are also associated with aggressive PCa [2,3]. Similar to many other cancers, the vast majority of these SNPs associated with PCa risk (approximately 98%) are located in noncoding regions, out-with exons, and hence do not act via altering protein-coding sequences [4,5]. Previous studies have shown that these noncoding risk SNPs are, however, significantly enriched in cis-regulatory elements (CREs) [5-7]. In addition, whole-genome sequencing (WGS) and exome sequencing studies have indicated that PCa is characterized by relatively low mutation rates but higher rates of recurrent genomic rearrangements [8], that can promote the upregulation of transcription factor genes.

The most well-characterized transcription factor associated with PCa is the androgen receptor (AR), which is known to be essential for the normal development of the prostate gland and involved in many aspects of PCa development including the progression to lethal metastatic castration-resistant prostate cancer (mCRPC) [9]. Emerging evidence has also implicated important roles for two other transcription factors: CCCTC-binding factor (CTCF) and forkhead box protein A1 (FOXA1). A CRISPR/Cas9 knockout screen for essential transcription factor binding sites found 10,000 binding sites for CTCF and FOXA1 that were essential for gene expression in breast and prostate cancer cells [10]. The FOXA1 binding sites were generally enhancers where FOXA1 acted with other transcription factors, such as AR, to

orchestrate the expression of nearby genes; whereas the CTCF binding sites fell into two classes: either acting in a similar fashion as enhancers regulating gene expression or a second class forming critical elements in chromosome organisation [10].

CTCF is a ubiquitously expressed, evolutionarily conserved, 11-zinc finger transcription factor that binds to more than 20,000 DNA loci in the human genome [11]. It is involved in both transcriptional activation and repression, DNA methylation, insulating, imprinting, DNA-loop formation, telomere maintenance and X chromosome inactivation [12-14]. CTCF is a critical chromatin-organizing factor that appears to be essential for the three-dimensional organization of chromatin due to its role in determining inter- and intra-chromosomal interactions [13,14]. Chromatin loops can be formed by CTCF binding to distant pairs of regulatory elements resulting in the bringing together, or splitting apart, of a distal regulatory element, such as an enhancer, with a promoter to elicit changes in gene expression.

FOXA1 is an evolutionarily conserved, winged-helix, pioneer factor that induces open chromatin conformation to facilitate the binding of other transcription factors such as the AR to bind to response elements and promote gene transcription [15,16]. FOXA1 and the AR co-occupy distant regulatory elements, such as enhancers, to regulate gene transcription [9,17]. In the prostate FOXA1 plays a critical role in the development of the gland where it maintains the differentiation of prostatic cells and is essential for prostate glandular morphogenesis [18,19]. The profile of androgen receptor binding sites in prostate epithelial cells is determined by FOXA1 and this is perturbed in prostate cancers [20]. A comprehensive molecular analysis of 333 primary prostate carcinomas, as part of The Cancer Genome Atlas (TCGA), revealed that FOXA1 mutant tumors were one of seven molecular classes of primary prostate cancer based on distinct oncogenic drivers [21]. The FOXA1 subtype tumors were observed to be amongst those with the

highest expression of AR-induced transcripts [21]. FOXA1 is the most enriched transcription factor found within prostate cancer-specific enhancer-promoter loop anchors [22]. FOXA1 has also been reported to be the third most frequently mutated gene in PCa [23]. Although the presence of FOXA1 appears to be obligatory for AR transcriptional activity, it does not seem to be essential for the expression of AR itself, as gene-deletion in mice has no effect on the expression or distribution of the AR [18]. However, a close relationship appears to exist between FOXA1 and AR in prostate tissue [24] and FOXA1 and AR bind together to form a complex that participates in AR nuclear localisation [25]. In addition, FOXA1 by binding to AR or by opening up chromatin structure may enable AR to transcriptionally regulate genes that do not possess a classical AR recognition sequence but have low-affinity or half-androgen response elements [26]. Furthermore, mutant-FOXA1 may play an important role in prostate cancer progression to androgen-independence by interacting with enhancers that are not dependent on AR, particularly for those genes that drive epithelial to mesenchymal (EMT) transition [27].

The insulin-like growth factors (IGF-I & IGF-II) are nutritionally regulated growth factors; with circulating levels of IGFs robustly associated with the risk of PCa [28] and Mendelian Randomization studies have implied that they play a causal role in PCa development [29]. IGFs also mediate some of the effects of lifestyle on PCa [30]. The essential role of CTCF in imprinting was first established for the imprinting of the IGF-II gene and the adjacent long non-coding RNA (lncRNA) gene H19 [31] and we have recently reported that this imprinting is metabolically regulated in prostate cancer cells [32]. Loss of imprinting (LOI) has been associated with many cancers, including PCa [33]. In addition, FOXA1 is an important mediator of IGF-signalling within cells [34]. We have also recently reported that EMT of prostate cancer cells, an important element of prostate cancer progression, is promoted by IGF-I, and by hyperglycemia, via a FOXA1 mediated induction of IGF-binding protein-2 (IGFBP-2) [35]. The intracellular signal

activated by IGFs is opposed by the phosphatase activity of the tumour-suppressor gene PTEN, which plays an important role in the progression of PCa and the development of mCRPC [36]. We have now examined associations between the transcription factors CTCF and FOXA1 and the AR and IGF-pathway in a cohort of men with localized prostate cancer.

Materials and Methods

The clinical prostate samples were collected for the Prostate cancer: Evidence of Exercise and Nutrition Trial (PrEvENT) [37] in Bristol, with full ethical approval (Ethics 14/SW/0056). Briefly, men from the National Health Service trust in the South West of England, UK diagnosed with localised PCa were recruited in two phases: 1) Baseline cohort of men scheduled for radical prostatectomy (RP) (N=96), 2) RCT cohort of men who underwent RP (N=81). Inclusion criteria included men over 18years of age, with written consent, and fit for the intervention. Men were consented to allow prostate tissue samples to be obtained from the prostate that was removed at the time of surgery. All samples were used and stored in accordance with the Human Tissue Act 2004 and the study was performed in accordance with the ICH GCP. Patient's clinical and pathological data including age at surgery, pre-surgery PSA levels, Gleason and histological grade and lymph node invasion were extracted and analysed anonymously from medical records and pathology reports for the purpose of this study.

Immunohistochemical (IHC) staining of tissue sections

Prostate tissue sections were formalin fixed and paraffin embedded (FFPE) and the sections examined were surplus to diagnostic requirements. Tissue was cut to 4μm thickness using a microtome (Leica) and collected and mounted on Tomo microscope slides (Matsunami, Bellingham, WA, USA). Slides were then stained on a Ventana BenchMark ULTRA™ machine (Roche, Oro Valley, AZ, USA) for peptides with the following antibodies: CTCF (1:1200, Novus Biologicals, Littleton, CO, USA), AR (1:150, Santa Cruz, Heidelberg, Germany), IGF1R (1:600, Cell Signaling, Hertfordshire, UK), PTEN (1:150, Dako, Santa Clara, CA, USA), phospho-mTOR(1:900, Cell Signaling), FOXA1 (1:450, Abcam,

Cambridge, UK), IGFBP-2(1:2000, Abcam) and Ki67 (1:300, Dako). Slides were scored by two pathologists using a modified version of the Allred system, combining the proportion of tissue stained (on a scale of 1–5) with the staining intensity (on a scale of 1–3) to give a score out of 8 [38].

Validation of the correlation analyses in prostate tissue

Results from the IHC staining were validated in publicly available datasets (cBioportal; Prostate Adenocarcinoma (TCGA, Pan Cancer cohort, N=489).

Results

FFPE sections of prostate tissues (70-92 depending on section availability) from the PrEvENT cohort were immunohistochemically stained with various antibodies and example sections were collated in Figure 1. The immunohistochemical staining for the chromatin-organizing factor CTCF was strongly correlated with staining for AR in benign prostate tissue ($r=0.51$, $p<0.001$, 95%CI: 0.3; 0.67) and a similar relationship was observed in the tumour tissue which approached statistical significance ($r=0.2$, $p=0.092$,95%CI: -0.04; 0.42) (Figure 2A). A stronger correlation between CTCF and AR was confirmed at the level of mRNA expression in the much larger cohort in the publicly available TCGA PanCancer Atlas database with mRNA data from 489 prostate adenocarcinomas ($r=0.53$, $p<0.0001$) (Figure 2B). CTCF was also strongly associated with staining for the IGF-IR in both benign ($r=0.49$, $p<0.0001$, 95%CI: 0.28;0.66)) and cancerous tissue ($r=0.54$, $p<0.0001$, 95%CI: 0.35;0.7)) (Figure 2C) and similarly strong correlation between CTCF and IGF-IR in TCGA PanCancer database was observed ($r=0.36$, $p<0.0001$) (Figure 2D). CTCF was also highly associated with staining for the tumour suppressor gene PTEN (benign, $r=0.4$, $p=0.001$, (95%CI: 0.17;0.59); cancer, $r=0.450$, $p<0.000$(95%CI: 0.23;0.63)); 1) (Table 1A). CTCF staining was significantly lower in PTEN-null tumours compared to those retaining PTEN staining($p<0.001$). CTCF also correlated with staining for phospho-mTOR (benign, $r=0.544$, $p<0.0001$ (95%CI:0.34;0.7); cancer, $r=0.346$, $p=0.003$ (95%CI: 0.11;0.54) (Table 1A), which is activated downstream of the IGF-IR.

Again, we confirmed these relationships in the TCGA database, at the mRNA expression level, where CTCF correlated with the IGF-IR ($r=0.35$, $p<0.001$) and mTOR ($r=0.31$, $p<0.001$); although the relationship with PTEN was not confirmed in this larger cohort (Table 1B).

CTCF has been implicated in the imprinting of the IGF-II/H19 loci, which has been associated with reciprocal expression of IGF-II and H19. In our cohort, in prostate tissue, both cancer and

benign, the expression of IGF-II mRNA was strongly positively correlated with H19 lncRNA(benign, r=0.67, p<0.0001; cancer, r=0.74, p<0.0001) [32] the same relationship was also seen in white blood cells (WBCs) (r=0.445, p<0.0001). We confirmed a similar strong positive correlation between IGF-II and H19 expression in a much larger cohort in the TCGA database (n=488, r=0.60, p<0.0001). In addition, in our cohort, for each of these genes there was a good correlation between expression of the gene in the matched benign and cancer tissue from the same individual (IGF-II mRNA, r=0.32, p=0.002; H19 lncRNA, r=0.35, p=0.0003); although there was no relationship found between expression in the prostate and in WBCs for either gene. No relationship was seen between the imprinting status and expression of IGF-II. In addition, there was no relationship between the circulating concentration of IGF-II peptide and either tissue IGF-II peptide staining or IGF-II mRNA expression in the prostate. There was, however, some consistency in relative imprinting status of the IGF-II locus within individuals between cancer, benign tissue and WBCs (benign v cancer, r=0.47, p=0.001; benign v WBCs r=0.39, p<0.001; cancer v WBCs r=0.43, p<0.001). Staining for CTCF was found to be strongly positively correlated with both IGF-II mRNA expression (r=0.38, p=0.002) and H19 lncRNA (r=0.27, p=0.03) only in the cancer tissue but not in benign tissue or in the white blood cells.

The immunohistochemical staining for the pioneer factor FOXA1 was also correlated with staining for AR in both benign and cancer tissue, although these relationships were not quite statistically significant at the formal level (benign, r=0.17, p=0.098(95%CI: -0.04; 0.36); cancer r=0.18, p<0.082 (95%CI: -0.03; 0.37) (Figure 3A)). FOXA1 also correlated with staining for the IGF-IR (benign, r=0.21,p=0.057 (95%CI: -0.01; 0.42); cancer, r=0.44,p<0.0001(95%CI: 0.23; 0.62))(Figure 3B), with staining for IGFBP-2(benign, r=0.24,p=0.017 (95%CI: 0.04;0.42); cancer, r=0.22,p=0.031(95%CI: 0.2;0.4)) and with

staining for activated phosphor-mTOR(benign, r=0.347,p=0.003 (95%CI: 0.12;0.54); cancer, r=0.255,p=0.03(95%CI: 0.02;0.46))(Table 1C).

[A]

	CTCF	r (95% CI) N	p value
PTEN	Benign	0.4 (0.17; 0.59) 71	<0.001
	Cancer	0.45 (0.23; 0.63)71	<0.001
mTOR	Benign	0.54 (0.34; 0.7)70	<0.0001
	Cancer	0.35 (0.11; 0.54)70	0.003

[B]

	CTCF	
	r	p value
IGF1R	0.35	<0.001
mTOR	0.31	<0.001

[C]

	FOXA1	r (95% CI) N	p value
IGFBP-2	Benign	0.24 (0.04; 0.42)98	0.017
	Cancer	0.22 (0.2; 0.4)98	0.031
mTOR	Benign	0.35 (0.12; 0.54)73	0.003
	Cancer	0.25 (0.02; 0.46)73	0.03

Table 1 [A]: Correlation between tissue-based CTCF and PTEN or mTOR [B**]**: Correlation between tissue-based FOXA1 and IGFBP-2 or mTOR **[C]**: Correlation between mRNA expression of CTCF and IGF1R or mTOR from TCGA database.

Again, we confirmed these relationships in the TCGA database, at the mRNA expression level, where FOXA1 correlated with AR (r=0.37,p<0.0001)(Figure 3B) and the IGF-IR (r=0.32, p<0.0001)(Figure 3D); however, we found no support for relationships between FOXA1 with

expression of IGFBP-2 and mTOR, although we did observe that expression of FOXA1 was inversely correlated with that of IGF-II($r=-0.40, p<0.001$) in this larger cohort.

The immunohistochemical staining for the IGF-IR was strongly correlated with staining for AR in benign prostate tissue ($r=0.46$, $p<0.0001$) and in the tumour tissue ($r=0.296$, $p<0.01$) Figure 4A) and the mRNA expression of these two genes were also strongly related in the TCGA database ($r=0.41$; $p<0.001$) (Figure 4B).

In relation to Gleason grade, the only factors that we found to be related were tissue staining for Ki67($p=0.013$) and IGFBP-2($p=0.004$). However, due to trials selection criteria to include men with localized prostate cancer, there was limited range of Gleason grading in our cohort with the vast majority being Gleason grade of 7 or less which would have limited the statistical power to observe such associations. When comparing Gleason grade 3+3 vs 4+3, we observed statistically significant differences with AR($p=0.029$) and Ki67($p=0.05$).

Discussion

Evidence from genetic studies indicates that PCa is characterized by relatively low mutation rates but higher rates of recurrent genomic rearrangements [8]; most of which promote the upregulation of transcription factor genes. Consistent with this, we have found that a critical chromatin-organizing factor, CTCF, and an important pioneer factor that induces open chromatin conformation, FOXA1, are both strongly related to factors in the androgen- and IGF-pathways known to be important in the development of PCa.

We found that the protein abundance of CTCF was positively related to that of the AR and that of the IGF-IR, PTEN and phosphor-mTOR in our cohort of prostate cancers. The relationships between CTCF and AR, IGF-IR and mTOR were confirmed at the level of mRNA expression in the publicly available TCGA database. Several lines of evidence have indicated that CTCF may play an important role in PCa [39]. GWAS have indicated that SNPs in the CTCF region were associated with PCa risk [40]. Cell culture studies have demonstrated that gene-silencing of CTCF affects the proliferation, migration, and invasion of prostate cells [41]. An immunohistochemistry study of CTCF expression in a tissue microarray containing 17,747 prostate cancers found that CTCF expression was significantly associated with advanced pathological tumor stage, high Gleason grade, nodal metastasis, and early biochemical recurrence; implicating an important for CTCF in PCa progression [42]. We found no relationship between CTCF staining and Gleason grade, but most tumours examined were Gleason grade of 7 or less and we therefore had limited statistical power to detect any association.

We found a weak relationship between that the protein abundance of FOXA1 and AR in our small cohort but a much stronger relationship between their mRNA expression was found in the larger TCGA database. A strong positive association between staining for FOXA1 and AR has previously been reported [43,44]. We also found that staining for FOXA1 correlated with that

for the IGF-IR and this was supported by a similar relationship between their mRNA expression in the TCGA database. The protein staining and mRNA expression of the IGF-IR were also strongly related to that of the AR; confirming that the IGF-IR is an AR regulated gene [45]. The effect of androgens on IGF-IR expression, however, does not appear to be a direct genomic effect of AR interacting with the IGF-IR promoter but was shown to be due to AR binding to c-Src with a resultant activation of cytoplasmic kinases that lead to stimulation of IGF-IR promoter activity [46]. The correlations between FOXA1 and the IGF-IR that we observed therefore probably do not reflect FOXA1 opening up the promoter region of IGF-IR for direct AR binding, as occurs for many genes [26,47], but could reflect an effect of FOXA1 to enable the binding of other transcription factors activated by these cytoplasmic kinases stimulating IGF-IR expression. The correlations between FOXA1 and the IGF-IR could also be due to the IGF-IR regulating FOXA1 as activation of IGF-IR has been reported to increase the stability of FOXA1 protein expression in breast cancer cells [34]. There appears to be a close inter-relationship between FOXA1, the AR and the IGF-IR in the prostate with FOXA1 being critical to the effects of the AR and the IGF-IR that can both drive prostate cancer progression; at least in part via the promotion of EMT [35].

While FOXA1 opens the chromatin for AR binding, CTCF can act to organize long-range chromatin architecture, either facilitating AR and FOXA1 binding or acting as an insulator to block other AR target genes [48]. In addition, CTCF plays a critical role in the IGF-axis with CTCF facilitating an intra-chromosomal loop that prevents enhancers binding to the maternal allele and facilitates the paternal imprinting of the IGF-II gene but, in a reciprocal manner permits expression of the maternal allele of the adjacent H19 gene [49]. In our cohort, however, we found that CTCF, IGF-II and H19 were all consistently positively correlated in both benign and malignant prostate tissue and, also in white blood cells and in the TCGA database the

expression of IGF-II and H19 were again strongly positively correlated. There was also consistency with the imprinting status of the IGF-II/H19 locus across benign and malignant prostate tissue and white blood cells within individuals. However, there was no relationship between imprinting status and IGF-II expression or between IGF-II expression in the prostate and that in white blood cells or with circulating IGF-II peptide levels. These results suggest that there is concordant regulation of IGF-II, H19 and CTCF and the imprinting status that is maintained systemically and also maintained within malignant prostate tissue. However, the expression of IGF-II in the prostate is tissue specific and distinct from that in white blood cells and from the determinants of circulating IGF-II levels. In many human tissues imprinting may play a minor role in the expression of IGF-II but a common enhancer shared by both IGF-II and H19 may be a more important determinant of the expression of both genes [50] and this may explain the concordance in expression.

Conclusions

Our data are consistent with a model in which CTCF acts via organising long-range chromatin architecture, determining which genes are accessible for AR binding and FOXA1 that interacts with the DNA to generate open chromatin. This facilitates AR binding to response elements in gene promoters, with the IGF-axis interacting with androgens to regulate, and be regulated by, each of these transcription factors. These findings emphasise the close and complex links between the endocrine controls, well known to play an important role in in prostate cancer, and the transcription factors implicated by the recent genetic evidence.

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Ethics approval statement

The clinical prostate samples were collected for the Prostate cancer: Evidence of Exercise and Nutrition Trial (PrEvENT) in Bristol, with full ethical approval (Ethics 14/SW/0056).

Contributorship Statement

RB, KB, CP, JH, RM, JL, LM, JO, AK & ER contributed to the planning of the study, RB, KB, GK, AS & PG conducted the work, RB & KB acquired and analysed the data, RB, KB, CP & JP interpreted the data, and all authors read and approved the final draft of the paper.

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Figure Legends

Figure 1. Immunohistochemical staining of IGFBP-2 benign (A) and cancer (B), CTCF benign (C) and cancer (D), FOXA1 benign (E) and cancer (F), PTEN benign (G) and cancer (H), IGF1R benign (I) and cancer (J), AR benign (K) and cancer (L), pmTOR benign (M) and cancer (N), Ki67 benign (O) and cancer (P).

Figure 2. Correlation between tissue-based AR and CTCF (A), IGF-IR and CTCF (C) peptide abundance from PrEvENT cohort. Size of dot and shadow represents the number of overlapping points. Correlation between AR and CTCF (B), IGF-IR and CTCF (D) mRNA expression from TCGA cohort.

Figure 3. Correlation between tissue-based FOXA1 and AR (A), FOXA1 and IGF-IR (C) peptide abundance from PrEvENT cohort. Size of dot and shadow represents the number of overlapping points. Correlation between FOXA1 and AR (B), FOXA1 and IGF-IR (D) mRNA expression from TCGA cohort.

Figure 4. Correlation between tissue-based AR and IGF-IR (A) peptide abundance from PrEvENT cohort. Size of dot and shadow represents the number of overlapping points. Correlation between AR and IGF-IR (B) mRNA expression from TCGA cohort.