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The kallikrein 14 gene is down-regulated by androgen receptor signalling and harbours genetic variation that is associated with prostate tumour aggressiveness

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Abstract

Kallikrein 14 (KLK14) has been proposed as a useful prognostic marker in prostate cancer, with expression reported to be associated with tumour characteristics such as higher stage and Gleason score. KLK14 tumour expression has also shown the potential to predict prostate cancer patients at risk of disease recurrence after radical prostatectomy. The KLKs are a remarkably hormone-responsive family of genes, although detailed studies of androgen regulation of KLK14 in prostate cancer have not been undertaken to date. Using in vitro studies, we have demonstrated that unlike many other prostatic KLK genes that are strictly androgen responsive, KLK14 is more broadly expressed and inversely androgen regulated in prostate cancer cells. Given these results and evidence that KLK14 may play a role in prostate cancer prognosis, we also investigated whether common genetic variants in the KLK14 locus are associated with risk and/or aggressiveness of prostate cancer in approximately 1200 prostate cancer cases and 1300 male controls. Of 41 single nucleotide polymorphisms assessed, three were associated with higher Gleason score (≥7): rs17728459 and rs4802765, both located upstream of KLK14, and rs35287116, which encodes a p.Gln33Arg substitution in the KLK14 signal peptide region. Our findings provide further support for KLK14 as a marker of prognosis in prostate cancer.

Keywords: androgen regulation; Gleason score; kallikreins; KLK14; prostate cancer; single nucleotide polymorphisms (SNPs).

Introduction

The kallikrein (KLK) family of serine peptidases are a prominent group of prostatic proteins. In the normal prostate, KLKs are secreted into seminal plasma where they act in an enzyme cascade to degrade the seminal clot (Veveris-Lowe et al., 2007; Emami and Diamandis, 2008). In prostate cancer, KLKs are over-expressed and aberrantly secreted into the tumour microenvironment and, ultimately, the bloodstream. This makes the KLKs not only potential functional mediators of tumour progression but also a rich source of biomarkers.

Like several other KLKs, KLK14 has been shown to be aberrantly expressed in hormone-dependent cancers of the breast, ovary and prostate and has been proposed as a useful prognostic marker (Mavridis and Scorilas, 2010). There is some evidence to support a role for KLK14 in prostate cancer risk and aggressiveness. KLK14 was found to be over-expressed in prostate tumours vs. matched normal tissue (Yousef et al., 2003b). Although Rabien et al. (2008) reported that KLK14 mRNA and protein were not differentially expressed between prostate cancer and normal tissue, this same study showed that KLK14 tumour protein expression was associated with tumour pathological characteristics such as higher stage and higher Gleason score, and had potential to predict prostate cancer patients at risk of disease recurrence [as defined by prostate-specific antigen (PSA) relapse] after radical prostatectomy. KLK14 tumour protein expression was also reported to be associated with higher Gleason score by Yousef et al. (2003b), and KLK14 has been shown to be elevated in serum from prostate cancer patients when compared with healthy males (Borgono et al., 2007). Given the recognition that genetic variation can be associated with altered protein expression and prostate cancer risk, as exemplified by...
the discovery that a single nucleotide polymorphism (SNP) in
KLK3 is associated with PSA levels and prostate cancer risk
(Eeles et al., 2008; Kote-Jarai et al., 2011; Parikh et al., 2011),
altogether these results indicate a need to further investigate
KLK14, and the SNPs within this gene, as a potential prostate
cancer biomarker.

Androgens are critically involved in prostate cancer pro-
gression, with androgen deprivation therapy being a key
therapeutic regimen for advanced disease. The KLKs are a
remarkably hormone-responsive family of genes, especially
KLK2 and KLK3, which are considered to be prototypical
androgen-regulated genes (Lawrence et al., 2010), and are
only expressed in androgen-responsive prostate epithelial
cells. Several studies have shown that KLK14 mRNA expres-
sion in breast and ovarian cancer cell lines is stimulated by
steroid hormones, including androgens, oestrogens, and pro-
gestins, although these effects are more subtle at the protein
level (Borgono et al., 2003; Yousef et al., 2003a; Paliouras
and Diamandis, 2007, 2008b; Shaw and Diamandis, 2008).
In prostate cancer cells, KLK14 is ubiquitously expressed at
low levels, and is not correlated with the androgen receptor
(AR) status of cell lines (Lawrence et al., 2010); however,
detailed studies of androgen regulation of KLK14 have not
been undertaken to date. In this study, we aimed to examine
androgen regulation of KLK14 expression in prostate cancer
cells with a goal of better understanding the reported associa-
tion with prostate cancer progression (Yousef et al., 2003b;
Rabien et al., 2008).

In parallel, we investigated the concept that common
genetic variation (SNPs) in the KLK14 gene may affect
KLK14 expression and hence alter prostate cancer risk and/or
the likelihood of more aggressive prostate tumours in carriers
by assessing 41 KLK14 gene region SNPs (±10 kb) in our
large prostate cancer case series and male controls.

Results

We have previously shown that KLK14 mRNA is widely
expressed in prostate cell lines, unlike KLK2 and KLK3, which
are only expressed in androgen-responsive cells (Lawrence
et al., 2010). Using Western blots, we observed that KLK14
protein (Figure 1) has the same expression profile. KLK14
expression was not only in LNCaP, 22Rv1 and MDA-PCa-2b
cells, which are all androgen responsive, but also in PC3 and
DU145 cells, albeit at lower levels, which lack the AR.

The broad expression profile of KLK14 implies that it
does not depend on AR signalling. To confirm this obser-
vation, increasing concentrations of the synthetic androgen
R1881 were added to LNCaP cells, a model of androgen-
dependent prostate cancer that resembles our patient cohort.
Surprisingly, KLK14 mRNA expression was significantly
down-regulated by treatment with 1 and 10 nM R1881 (Figure
2A). A time course of R1881 treatment showed that KLK14
mRNA was significantly down-regulated at 24 h (Figure 2B);
however, the decrease in KLK14 protein was not apparent
until 7 days of R1881 treatment (Figure 2C). To confirm these
changes, we investigated whether decreasing AR activity has

the opposite effect on KLK14 expression. LNCaP cells were
deprived of hormones by being cultured in medium contain-
ing charcoal-stripped serum (CSS). After 4 days, there was
an increase in both KLK14 mRNA (Figure 2D) and protein
(Figure 2E) levels. As expected, there was also a decrease in
AR protein in hormone-deprived LNCaP cells (Figure 2E).
When AR activity was blocked pharmacologically, using
the AR antagonist bicalutamide, KLK14 expression was
restored compared with R1881 treatment alone (Figure 2F).
Furthermore, KLK14 expression was actually stimulated with
100 μM bicalutamide. These changes are specific to AR activ-
ity because siRNA knockdown of the AR also rescues KLK14
expression (Figure 2G). These observations show that
KLK14 is moderately, but significantly, down-regulated by AR
signalling, and is therefore differentially regulated compared
with other prostatic KLKs.

Given that KLK14 is consistently expressed in prostate
cancer cells and significantly upregulated in a hormone-
deprived milieu (CSS) or on anti-androgen treatment (bicalu-
tamide), and may play a role in prognosis, we investigated
whether SNPs within the KLK14 gene are associated with
the occurrence and aggressiveness of prostate cancer. Six
SNPs were found to be non-polymorphic in our sample group
(Table 1). Results of analyses of the remaining 35 KLK14
SNPs and risk of prostate cancer are displayed in Table 2. No
KLK14 SNPs were statistically significantly associated with
prostate cancer risk or tumour aggressiveness after Bonferroni
correction (p≤0.0014). SNP rs66613646 was associated with
a modest increased risk of prostate cancer at the p≤0.05
level [odds ratio (OR) 1.19, 95% confidence interval (CI)
1.03–1.38, pmeta=0.022]; however, there was no association
with risk for tagSNP rs867192 (OR 1.05, 95% CI 0.91–1.22,
pmeta=0.521; Table 1), found by analysis of our control dataset
to be in high linkage disequilibrium (LD) with rs66613646
(r2=0.79; data not shown).

In relation to the possible role of KLK14 SNPs in tumour
aggressiveness, three SNPs displayed p-values of ≤0.05
when Gleason scores <7 were compared with ≥7 (Table 3).
Rs17728459 located approximately 9 kb upstream of KLK14
was associated with a protective effect, with an OR of 0.33
(95% CI 0.15–0.73, pmeta=0.006). A similar result was also

Figure 1 KLK14 is ubiquitously expressed in prostate cancer
cells.
Western blots of KLK14 expression in LNCaP, 22Rv1, PC-3, DU145
and MDA-PCa-2b prostate cell lines. KLK14 was detected as an
approximately 22-kDa band. GAPDH was used as a loading control.
Representative data from two experiments are shown.
Figure 2  KLK14 is down-regulated by androgens.
(A) KLK14 expression in LNCaP cells treated with 0–10 nM R1881 for 24 h. Data are from qPCR analysis and expressed as average fold changes vs. the ethanol control (n=3, asterisks indicate significant difference in the fold change relative to 0 nM). (B) Relative KLK14 expression in LNCaP cells treated with 1 nM R1881 for up to 24 h as measured using qPCR. Data represent average fold changes vs. the corresponding ethanol control for each time point (n=2, asterisks indicate significant differences between R1881 and the control at each time point). (C) Western blots of KLK14 protein expression in LNCaP cells treated with 0–10 nM R1881 for 1 day or 1 nM R1881 for 3 and 7 days. DU145 cells (DU), which express lower levels of KLK14, were used as control. GAPDH was used to confirm equal protein loading. Representative data from two (7 days) or three (1 and 3 days) separate experiments are shown. (D) qPCR data of KLK14 expression in LNCaP cells cultured in medium with CSS for up to 10 days. Results represent average fold changes compared with day 0 (n=3, asterisks indicate significant difference in fold change relative to day 0). (E) Western blots of KLK14 and GAPDH expression in hormone-deprived LNCaP cells. Representative data from two independent experiments are shown. (F) KLK14 expression in LNCaP cells treated with 1 nM R1881 and 0–100 μM bicalutamide (BIC). Relative fold changes vs. R1881 alone were measured using qPCR (n=3, asterisks indicate significant difference in fold change relative to R1881 alone). LNCaP cells treated with and without 1 nM R1881 were also compared. (G) qPCR data of AR (white bars) and KLK14 (grey bars) expression in LNCaP cells transfected with control or AR-specific siRNA and treated for 48 h with 1 nM R1881. Data are normalised to the mock transfection control (n=3, general linear model, asterisks indicate significant difference between control and AR-specific siRNA). For all experiments, ▲ p<0.05, ● p<0.01, ★ p<0.001.

seen when ‘extreme’ Gleason scores were compared (≤6 vs. ≥8, Table 4), although sample numbers were substantially decreased and the CI overlapped unity. Rs4802765, located around 2 kb upstream of KLK14, displayed a borderline association with increased risk of tumour aggressiveness (OR 1.31, 95% CI 1.00–1.72, p=0.050); however, this effect was not reflected in the Gleason score ≤6 vs. ≥8 analysis, despite the high minor allele frequency of 0.33. Rs35287116 displayed an association with increased tumour aggressiveness (OR 1.28, 95% CI 1.06–1.56, p=0.012), which was similarly reflected when analyses were restricted to Gleason score ≤6 vs. ≥8 (Table 4; OR 1.38, 95% CI 1.03–1.85, p=0.030). Studies in our control set revealed rs35287116, encoding a glutamine to arginine amino acid substitution at position 33 in the signal peptide region of KLK14 (Clements et al., 2004), was correlated with rs10500304 (r2=0.87), and this SNP showed comparable, although not significant, results (OR 1.18, 95% CI 0.95–1.46, p=0.130). In addition, SNPs rs73051038 exhibited an association with increased tumour aggressiveness only when analyses were restricted to ‘extreme’ Gleason scores, with OR for Gleason score ≤6 vs. ≥8 of 1.94 (95% CI 1.10–3.40, p=0.021), in the same

Table 1  Rs IDs found to be very rare or non-polymorphic in this study.

<table>
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<tr>
<th>Rs ID</th>
<th>Ref. allele</th>
<th>Controls (n)</th>
<th>Cases (n)</th>
<th>Samples detected (n)</th>
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<td>1228</td>
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<td>1236</td>
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</table>

ID, identifier; Ref., reference; n, number.
direction as results for Gleason score <7 vs. ≥7 (OR 1.38, 95% CI 0.91–2.07, \( p_{\text{trend}} = 0.126 \)).

**Discussion**

Since KLK14 was first cloned in 2001, the prostate has been known to be a prominent site of KLK14 gene expression (Hooper et al., 2001; Yousef et al., 2001). Yet, relatively little is known about the expression profile and transcriptional regulation of KLK14 in the prostate. In this study, we showed that KLK14 is more broadly expressed across androgen-responsive and -insensitive cell lines and is down-regulated by AR signalling.

Considering that prostatic KLKs such as KLK2 and KLK3 are classical AR-activated genes (Lawrence et al., 2010), the observation that androgens suppress KLK14 expression was unexpected. The down-regulation of KLK14 was modest, but consistent, with a 40–75% decrease depending on the experiment. There was a much more rapid decrease in KLK14 mRNA compared with protein levels. This may be due to the relative instability of KLK14 mRNA, which has a half-life of <6 h (M. Lawrence, unpublished observations). Nevertheless, as has also been noted in studies that measured secreted KLK14 levels with ELISAs (Paliouras and Diamandis, 2007, 2008a), changes in KLK14 mRNA and protein levels are correlated. Moreover, the changes we observed are specific because KLK14 levels increase with extended hormone deprivation, bicalutamide treatment and AR knockdown. The modulation, but not complete repression, of KLK14 expression is also consistent with its ubiquitous expression among prostate cancer cell lines. Intriguingly, these results may be tissue specific; previous studies have shown that KLK14 is up-regulated by androgens in a range of breast and ovarian cancer cell lines (Borgono et al., 2003; Yousef et al., 2003a; Paliouras and Diamandis, 2007, 2008b). Therefore, whether KLK14 is activated or repressed by androgens may depend on crosstalk between the AR and other signalling pathways in different cell types.

# Table 2

<table>
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<tr>
<th>KLK14 SNP</th>
<th>Ref. allele</th>
<th>Alt. allele</th>
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<th>Cases (n)</th>
<th>OR (95% CI)</th>
<th>( p_{\text{trend}} )</th>
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SNP, single nucleotide polymorphism; Ref., reference; Alt., alternate; n, number; OR, odds ratio; CI, confidence interval. Bold font indicates SNP displaying a \( p \)-value \( \leq 0.05 \).
<table>
<thead>
<tr>
<th>SNP</th>
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<th>GS ≥ 7 (n)</th>
<th>OR (95% CI)</th>
<th>p &lt;br&gt;value</th>
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SNP, single nucleotide polymorphism; GS, Gleason score; n, number; OR, odds ratio; CI, confidence interval.
Bold font indicates SNPs displaying a p-value ≤ 0.05.
*SNP too infrequent in these groups to calculate OR (95% CI).

The repressive actions of the AR are well recognised but poorly understood. Studies have noted that slightly fewer genes are repressed by androgens than stimulated (Clegg et al., 2002; Bolton et al., 2007; Prescott et al., 2007). Like KLK14, most of these genes are moderately down-regulated rather than completely repressed (Kojima et al., 2006; Prescott et al., 2007). One potential mechanism is that AR actively inhibits target gene expression by recruiting co-repressor proteins such as NCoR and SMRT. This is unlikely for KLK14 because its expression is rescued by bicalutamide, which maintains the ability of the AR to interact with co-repressor and bind to DNA (Hodgson et al., 2007; Prescott et al., 2007). The AR also down-regulates gene expression without binding to target promoters. It can compete for limited pools of co-activators or form inhibitory interactions with other transcription factors such as NFκB, Sp1, AP-1, cJun and ATF2 (Grosse et al., 2012). Therefore, the KLK14 promoter should be further characterised to help determine the mechanism of AR-mediated repression.

The differential androgen regulation of KLK14 compared with other prostatic KLKs has important implications for their relative expression profiles in prostate cancer. AR-activated genes such as KLK2 and KLK3 are generally down-regulated as the primary tumour de-differentiates and are then further reduced in metastases (Clegg et al., 2002; Bolton et al., 2007; Prescott et al., 2007). In patients that undergo androgen deprivation therapy, AR activated genes are initially down-regulated, but are then re-expressed in castrate-resistant disease (Holzbeierlein et al., 2004). Although KLK3 expression generally follows these trends, it is highly heterogeneous after hormone deprivation therapy as tumours proceed towards
castration resistance (Mostaghel et al., 2007). This variability may be due to the different ways that prostate cancer cells adapt to castrate androgen levels. Although the effect of hormone ablation on KLK14 expression has not yet been reported, other genes that are repressed by androgens, such as IGFBP3 and relaxin, are initially up-regulated by hormone deprivation therapy, but then decrease in castrate-resistant prostate cancer (Kojima et al., 2006; Thompson et al., 2006). This suggests that KLK3 and KLK14 may have opposite expression profiles. If this is the case, the ratio of KLK3 to KLK14 expression may be a useful measure of AR activity that overcomes some of the variability between patients. Therefore, further analysis of the relative expression profiles of KLK3, KLK14 and other prostatic KLKs is warranted.

In parallel to our investigation of the androgen regulation of KLK14 expression, we performed a comprehensive investigation of the role of common genetic variations in the KLK14 gene in prostate cancer risk and/or tumour aggressiveness by assessing the majority of KLK14 SNPs that have not been covered by previously performed genome-wide association (GWA) studies. Our study of over 1200 cases and 1300 male controls identified no SNPs to be associated with prostate cancer predisposition, but revealed several KLK14 SNPs to be potentially associated with tumour aggressiveness. To the best of our knowledge, only one other study has specifically examined the role of KLK14 SNPs in prostate cancer aside from genome-wide investigations. Analogous to our findings, the study by Klein et al. (2010) found none of the KLK14 SNPs assessed (including rs2569491, rs35287116, rs7260199, rs7259389 and rs73596529) in our study to be associated with prostate cancer risk in the Cancer Prostate in Sweden (CAPS) 1 sample set of over 1400 cases and 700 controls. The study of Klein et al. did not assess the relation of KLK14 SNPs with tumour aggressiveness.

In our study of approximately 1200 cases, two SNPs upstream of KLK14 were found to be associated with tumour aggressiveness, rs17728459 with decreased aggressiveness and rs4802765 with more aggressive tumours. Neither were located in known or predicted hormone response elements (Sandelin and Wasserman, 2005; Kennedy et al., 2010), although preliminary bioinformatic analysis of the potential effects of rs4802765 T>C on transcription factor binding sites (Grabe, 2000) predicted that the presence of the C allele introduces two Sp1 sites and an NF-1 site. Interestingly, Sp1 regulates the expression of the AR and KLK3 genes, and Sp1 expression has been found to be elevated in prostate cancer and associated with prognosis (Sankpal et al., 2011). In addition, a GWAS-identified prostate cancer-associated SNP near the NKX3.1 gene (Eeles et al., 2009) has recently been shown to alter the binding of Sp1 (Akamatsu et al., 2010). We also identified SNP rs35287116 in the coding region of KLK14 to be associated with increased tumour aggressiveness. Rs35287116 encodes the non-synonymous substitution p.Gln33Arg in the signal peptide region (Clements et al., 2004), but is not predicted to alter cleavage of the signal peptide by SignalP 3.0 (Bendtsen et al., 2004) or PSORT II (Nakai and Horton, 1999) and is calculated to be tolerated by the bioinformatic algorithms SIFT (Ng and Henikoff, 2001) and PolyPhen-2 (Adzhubei et al., 2010). However, ESEFinder (Smith et al., 2006) predicts that rs35287116 may affect an exonic splicing enhancer site and hence alter splicing of KLK14.

Although the functional role of KLK14 in prostate cancer has not been investigated, the substrates that have been identified in biochemical studies could explain the association between KLK14 SNPs and tumour aggressiveness. KLK14 is normally secreted into seminal plasma (Borgono et al., 2007; Emami et al., 2008), but the breakdown of glandular architecture in prostate cancer likely leads to the accumulation of KLK14 in the tumour microenvironment. KLK14 may directly promote the migration and invasion of tumour cells by activating proteinase activated receptor 2 signalling (Oikonomopoulou et al., 2006; Stefansson et al., 2008; Gratio et al., 2011); degrading extracellular matrix proteins such as fibronectin, laminin and collagen I to IV (Borgono et al., 2007; Rajapakse and Takahashi, 2007); and activating enzyme cascades with KLK1-3, KLKS and KLK11 (Brattsand et al., 2005; Yoon et al., 2007; Emami and Diamandis, 2010). KLK14 also activates latent transforming growth factor β1 (Emami and Diamandis, 2010) and degrades insulin-like growth factor binding proteins 2 and 3 (Borgono et al., 2007), suggesting it affects the growth and differentiation of prostate cancer cells. Ultimately, however, the actions of KLK14 in the tumour microenvironment will also depend on the levels of other proteases that activate it, as well as serpins and metal ions that inhibit KLK14 enzyme activity.

To date, there has been little success in attempts to delineate, and then validate, the contribution of SNPs to prostate tumour aggressiveness (Duggan et al., 2007; Kader et al., 2009; Witte, 2009; Lubahn et al., 2010; Fitzgerald et al., 2011; Lin et al., 2011). There are several possible reasons for this (reviewed in Oon et al., 2011), such as the fact that one prostate can have multiple tumour foci that display different Gleason scores, and it is well known that there is much heterogeneity between tumours that are graded Gleason score 7. We attempted to circumvent some issues involving tumours with Gleason score 7 by restricting analyses to those patients with the more ‘extreme’ Gleason scores of ≤6 and ≥8. Although this depleted our sample set dramatically, several SNPs remain candidates for further investigation in larger sample sets. Unfortunately, long-term follow-up data were not available for our prostate cancer cases, so we could not investigate the role of KLK14 SNPs in prognosis at this time; however, this will be an important analysis in the future.

In summary, we have demonstrated that unlike other prostate-expressed KLK genes that are strictly androgen responsive, KLK14 is more broadly expressed and inversely androgen regulated, increased by anti-androgens and in an androgen-deprived milieu. This suggests that KLK14 expression is maintained throughout prostate cancer, including the initial stages of hormone deprivation, with subsequent proteolytic effects in the tumour microenvironment that promote tumour progression. In addition, our well-sized study suggests a contribution of SNPs in the KLK14 gene to the...
development of more aggressive prostate cancer, an interesting finding considering previous reports that KLK14 expression is a possible prognostic factor for tumour characteristics and progression-free survival. Validation of these results in much larger sample sets such as those available from the PRACTICAL prostate cancer consortium would highlight KLK14 SNPs as a possible prognostic marker to identify patients that may benefit from more immediate and proactive treatment of their disease.

Materials and methods

KLK14 expression and androgen regulatory studies

Cell culture and androgen treatments LNCaP, 22Rv1, PC3, DU145 and MDA-PCa-2b prostate cancer cell lines were acquired from the American Type Culture Collection (Manassas, VA, USA). All cells were grown in RPMI 1640 medium (Invitrogen, Mount Waverly, Australia) with 10% foetal calf serum (FCS, Invitrogen), 50 μg/ml penicillin G and 50 μg/ml streptomycin (Invitrogen), except for MDA-PCa-2b cells, which were cultured in BRFF-HPC1 medium (AthenaES, Baltimore, MD, USA) with 20% FCS and antibiotics. LNCaP cells were cultured in phenol red-free RPMI 1640 with 10% CSS for 72 h before androgen treatments. Cells were then treated with 0.01–10 nm R1881 (Perkin Elmer, Boston, MA, USA) or ethanol vehicle control for 24 h, unless otherwise stated. For hormone deprivation experiments, LNCaP cells were cultured in medium containing CSS for up to 10 days with medium changes after 4 and 7 days. For bicalutamide treatments, cells were pre-treated for 2 h with 0–100 μM bicalutamide (AstraZeneca, Brisbane, QLD, Australia) and then cultured for a further 24 h with 1 nm R1881 and bicalutamide. AR knockdown with siRNA was performed as previously described (Jia et al., 2006, 2008). LNCaP cells were transfected with an AR-specific or scrambled siRNA for 48 h using Oligofectamine (Invitrogen) and then treated for 48 h with 1 nm R1881.

RNA extractions and quantitative RT-PCR Total RNA was extracted using TRIzol and then treated with DNase I (Invitrogen). SuperScript III reverse transcriptase was used to synthesise cDNA extracted using TRIzol and then treated with DNase I (Invitrogen). LNCaP cells were cultured in phenol red-free RPMI 1640 with 10% CSS for 72 h before androgen treatments. Cells were then treated with 0.01–10 nm R1881 (Perkin Elmer, Boston, MA, USA) or ethanol vehicle control for 24 h, unless otherwise stated. For hormone deprivation experiments, LNCaP cells were cultured in medium containing CSS for up to 10 days with medium changes after 4 and 7 days. For bicalutamide treatments, cells were pre-treated for 2 h with 0–100 μM bicalutamide (AstraZeneca, Brisbane, QLD, Australia) and then cultured for a further 24 h with 1 nm R1881 and bicalutamide. AR knockdown with siRNA was performed as previously described (Jia et al., 2006, 2008). LNCaP cells were transfected with an AR-specific or scrambled siRNA for 48 h using Oligofectamine (Invitrogen) and then treated for 48 h with 1 nm R1881.

Protein extractions and Western blotting Cells were lysed in buffer containing 10 mm Tris (pH 8.0), 150 mm NaCl, 5 mm EDTA, 1% Triton X-100 and 1× complete protease inhibitor cocktail (Roche, West End, QLD, Australia), and total protein concentrations were measured using a Bicinchoninic Assay Kit (Pierce, Progen, Darra, QLD, Australia). Forty micrograms of each sample was separated using 14% sodium dodecyl sulphate-polyacrylamide gels. Membranes were probed with either a rabbit anti-KLK14 catalytic domain antibody [a gift from Preston Alexander, TriplePoint Biologies (Forest Grove, OR), and also available from Abcam, Cambridge, MA, USA] or a rabbit anti-GAPDH antibody (Abcam) diluted in Odyssey buffer (LI-COR Biosciences, Millennium Science, Surrey Hills, NSW, Australia). The blots were then incubated with a goat-anti-rabbit-680 (Invitrogen) fluorescent secondary antibody and imaged using a LI-COR Odyssey scanner. Odyssey software was used to adjust the brightness, contrast and intensity of images.

Analysis of KLK14 SNPs in prostate cancer cases and controls

Study subjects Study subjects have been described elsewhere (Baade et al., 2010; Lose et al., 2011). Briefly, from 2004 onwards, 1349 histopathologically confirmed prostate cancer cases were recruited through private and public urologists in Queensland, Australia, through three prostate cancer studies or resources: the Retrospective Queensland Study (n=154; Lai et al., 2007), the Prostate Cancer Supportive Care and Patient Outcomes Project (ProsCan, n=857; Baade et al., 2010) and from the Australian Prostate Cancer BioResource (APCB, n=338; http://www.appc RESOURCE. ORG.AU/index.html). Men presented to urologists with lower urinary tract symptoms and/or abnormal serum PSA, and 72% of cases possessed prostatic tumours of Gleason score 7 or above. Cases ranged in age at diagnosis from 36 to 88 years (median 63 years). Male controls (n=1405) with no self-reported personal history of prostate cancer were randomly selected from the Australian Electoral Roll and age-matched (in 5-year groups) and post-code matched to cases (n=569), or recruited through the Australian Red Cross Blood Services in Brisbane (n=836). Controls were not screened for PSA levels and analyses excluded 38 controls with age at interview <36 years (the age of the youngest case); included controls ranged in age at interview from 36 to 89 years of age (median 62 years). All participants had self-reported Caucasian ethnicity and gave written informed consent. The study protocol was approved by the Human Research Ethics Committees of the Queensland University of Technology, Queensland Institute of Medical Research, the Mater Hospital (for Brisbane Private Hospital), the Royal Brisbane Hospital, Princess Alexandra Hospital and the Cancer Council Queensland.

SNP selection and genotyping The KLK14 gene region used for SNP selection was chr19:56262966..56289314 (hg18), which encompasses the KLK14 gene ±10 kb. All SNPs in this region were extracted from National Center for Biotechnology Information (NCBI) dbSNP build 130 (Sherry et al., 2001), CHIPSNPper (Riva and Kohane, 2004) and the ‘ParSNPs’ database (Goard et al., 2007) and duplicates were removed. SNPs not classified as validated were removed, and validated SNPs were further investigated for occurrence in Caucasians using SPSmart (Amigo et al., 2008) and 1000 Genomes (2010). Additional SNPs excluded from investigation included all SNPs on the Illumina 550K, 610K and Omni1 wide genotyping chips and SNPs assessed in the Cancer Genetic Markers of Susceptibility (CGEMS) project (Yeager et al., 2007), unless there was evidence of association with prostate cancer by CGEMS (p<0.05). SNPs in high LD (r2≥0.80) with these excluded Illumina and CGEMS SNPs were also removed, determined by the SNP Annotation and Proxy Search program (SNAP) version 2.1 (Johnson et al., 2008) using HapMap release 22 (1000 Genomes data was not available at the time of initiation of this study). We then prioritised for genotyping all independent SNPs (r2<0.80) according to SNAP using HapMap release 22 data (n=42). As part of a previous study, we had also genotyped eight KLK14 tagSNPs (selected using HapMap data release 24/phase II, Nov 2008, NCBI build 36, dbSNP b126, using the Tagger program within Haploview v4.1; Barrett et al., 2005).

SNPs were genotyped using iPLEX Gold assays on the Sequenom MassARRAY platform (Sequenom, San Diego, CA, USA), as described previously (Lose et al., 2010). There were four negative (H2O) controls per 384-well plate, and quality control parameters included genotype call rates >95%, a combination of cases and controls on each plate, inclusion of 20 duplicate samples per 384-well plate (>5% of samples) with ≥98% concordance between duplicates and Hardy-Weinberg equilibrium p-values >0.05. Of a total of 50
KLK14 SNPs selected for investigation, six could not be designed for Sequenom assays, and after application of quality control parameters, 41 SNPs were successfully genotyped.

Statistical methods

Gene expression data were analysed using Predictive Analytics Software (PASW) Statistics version 17.0.2 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism. The significance of fold changes relative to the reference group was assessed by one-sample t-test. Differences of the fold changes between groups were assessed using one-way ANOVA, and when there was evidence of variance heterogeneity the Brown-Forsythe robust test (Brown and Forsythe, 1974) was used. General linear models were used to compare the fold changes relative to mock R1881 between the control and AR siRNA groups (Figure 2G). All error bars represent the standard error of the mean of biological replicates performed on separate occasions. p-Values are indicated by ** for p<0.05, *** for p<0.01, and **** for p<0.001.

PASW Statistics version 17.0.2 (SPSS Inc.) was used for genetic analyses. Genotype and allele frequencies were calculated for the patient and control groups. Comparisons of allele and genotype distribution and their association with prostate cancer susceptibility and clinical data were analysed under a linear model using logistic regression analysis and all analyses were adjusted for age (as a continuous variable). Prostate cancer cases with tumour Gleason scores ≥7 were classified as aggressive. Analyses were also performed comparing ‘extreme’ Gleason categories, ≤6 (maximum number of cases=334) vs. ≥8 (maximum cases=176).

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References


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