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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

Felicity Lose[1,2,a], Mitchell G. Lawrence[2,a,b], Srilakshmi Srinivasan[2], Tracy O'Mara[1,2], Louise Marquart[3], Suzanne Chambers[4–6], Robert A. Gardiner[6], Joanne F. Aitken[4,5], Amanda B. Spurdle[1], Jyotsna Batra[1,2], Judith A. Clements[2,]* and the Australian Prostate Cancer BioResource[2]

[1] Molecular Cancer Epidemiology Group, Genetics and Population Health Division, Queensland Institute of Medical Research, 300 Herston Road, Herston, Brisbane, QLD 4006, Australia

[2] Australian Prostate Cancer Research Centre-Queensland, Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, Brisbane, QLD 4059, Australia

[3] Statistics Unit, Queensland Institute of Medical Research, 300 Herston Road, Herston, Brisbane, QLD 4006, Australia

[4] Griffith Health Institute, Griffith University, Southport, Brisbane, QLD 4222, Australia

[5] Viertel Centre for Cancer Research, Cancer Council Queensland, Fortitude Valley, Brisbane, QLD 4004, Australia

[6] University of Queensland Centre for Clinical Research, Royal Brisbane Hospital, Herston, Brisbane, QLD 4029, Australia

* Corresponding author
e-mail: j.clements@qut.edu.au

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).

[a]These authors contributed equally to this study.
[b]Present address: Department of Anatomy and Developmental Biology, Monash University, Clayton, VIC 3800, Australia.

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

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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 progression, 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 expression in breast and ovarian cancer cell lines is stimulated by steroid hormones, including androgens, oestrogens, and progestins, 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 association 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 is expressed 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 observation, 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

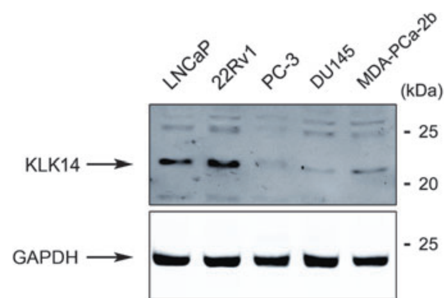

Figure 1 KLK14 is ubiquitously expressed in prostate cancer cells.
Western blots of KLK14 expression in LNCaP, 22Rv1, PC-3, DU145 and MDA-PCa-2b prostate cell lines. KLK14 was detected as an approximately 22-kDa band. GAPDH was used as a loading control. Representative data from two experiments are shown.

the opposite effect on *KLK14* expression. LNCaP cells were deprived of hormones by being cultured in medium containing 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 activity 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 (bicalutamide), 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 ($p \leq 0.0014$). SNP rs66613646 was associated with a modest increased risk of prostate cancer at the $p \leq 0.05$ level [odds ratio (OR) 1.19, 95% confidence interval (CI) 1.03–1.38, $p_{trend}=0.022$]; however, there was no association with risk for tagSNP rs867192 (OR 1.05, 95% CI 0.91–1.22, $p_{trend}=0.521$; Table 1), found by analysis of our control dataset to be in high linkage disequilibrium (LD) with rs66613646 ($r^2=0.79$; 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, $p_{trend}=0.006$). A similar result was also

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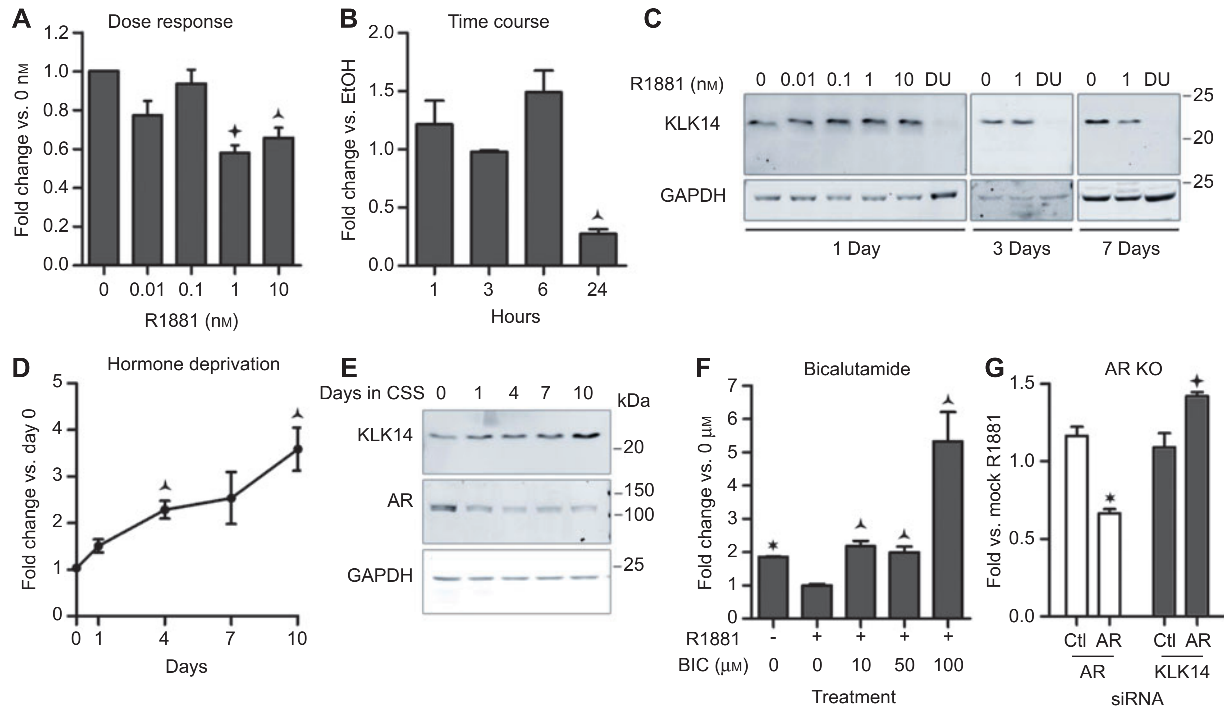

Figure 2 *KLK14* is down-regulated by androgens.
(A) *KLK14* expression in LNCaP cells treated with 0–10 nM R1881 for 24 h. Data are from qPCR analysis and expressed as average fold changes vs. the ethanol control (n=3, asterisks indicate significant difference in the fold change relative to 0 nM). (B) 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 R1181 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_{trend}=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_{trend}=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_{trend}=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 (r^2=0.87), and this SNP showed comparable, although not significant, results (OR 1.18, 95% CI 0.95–1.46, p_{trend}=0.130). In addition, SNP 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_{trend}=0.021), in the same

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

Rs ID	Ref. allele	Controls (n)	Cases (n)	Samples detected (n)
rs1880416	C	1213	1228	0
rs73596505	C	1266	1236	0
rs73596515	G	1272	1229	0
rs2569494	G	1215	1228	1
rs2569495	T	1215	1228	0
rs2691268	G	1272	1229	0

ID, identifier; Ref., reference; n, number.

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Table 2 Association of *KLK14* SNPs and prostate cancer risk.

KLK14 SNP	Ref. allele	Alt. allele	Controls (n)	Cases (n)	OR (95% CI)	p_{trend}
rs73596564	A	G	1072	1070	1.39 (0.48–4.02)	0.547
rs17728459	C	T	1094	1087	0.91 (0.55–1.51)	0.712
rs73596556	C	T	1210	1224	1.83 (0.61–5.48)	0.283
rs2691271	C	G	1255	1225	0.87 (0.75–1.01)	0.060
rs12984853	G	A	1262	1233	0.99 (0.88–1.11)	0.890
rs34974522	G	A	1204	1210	0.93 (0.83–1.04)	0.201
rs2691270	A	G	1258	1186	1.06 (0.95–1.19)	0.288
rs2569497	T	C	1214	1225	0.93 (0.81–1.07)	0.291
rs34368430	G	A	1214	1227	1.10 (0.97–1.24)	0.141
rs1880417	A	G	1272	1229	2.30 (0.21–25.46)	0.496
rs4802765	T	C	1265	1220	0.87 (0.75–1.02)	0.083
rs7259389	C	T	1249	1171	0.98 (0.84–1.15)	0.833
rs73596529	C	T	1212	1226	0.96 (0.82–1.14)	0.665
rs7260199	C	T	1205	1214	0.96 (0.85–1.08)	0.481
rs12609392	G	A	1206	1212	0.99 (0.88–1.11)	0.869
rs73051038	G	C	1266	1240	0.98 (0.79–1.22)	0.863
rs2072689	G	A	1309	1001	1.08 (0.96–1.21)	0.199
rs2235100	A	G	1296	994	1.01 (0.87–1.17)	0.934
rs2691244	A	G	1307	997	1.10 (0.98–1.25)	0.121
rs62114145	G	A	1252	1184	1.06 (0.91–1.23)	0.450
rs62114144	G	A	1202	1211	1.14 (0.98–1.33)	0.078
rs35287116	T	C	1264	1238	1.06 (0.94–1.18)	0.354
rs2569491	G	A	1254	1172	1.07 (0.94–1.21)	0.313
rs2569490	C	T	1183	1193	1.43 (0.61–3.36)	0.414
rs10500304	A	C	1296	995	1.08 (0.96–1.22)	0.218
rs10445581	G	C	1315	1003	1.05 (0.92–1.19)	0.495
rs867192	C	A	1291	977	1.05 (0.91–1.22)	0.521
rs11671942	C	T	1327	1002	0.96 (0.79–1.16)	0.680
rs11671800	C	A	1326	1000	1.06 (0.91–1.24)	0.441
rs66613646	**G**	**T**	**1195**	**1194**	**1.19 (1.03–1.38)**	**0.022**
rs2569487	G	A	1252	1183	0.93 (0.82–1.05)	0.251
rs2569485	T	C	1261	1237	1.02 (0.91–1.14)	0.708
rs7253072	C	T	1258	1185	1.06 (0.88–1.28)	0.520
rs7253169	G	A	1256	1227	1.02 (0.85–1.22)	0.831
rs2691215	A	T	1071	1068	0.97 (0.86–1.10)	0.632

SNP, single nucleotide polymorphism; Ref., reference; Alt., alternate; n, number; OR, odds ratio; CI, confidence interval.
Bold font indicates SNP displaying a p-value ≤0.05.

direction as results for Gleason score <7 vs. ≥7 (OR 1.38, 95% CI 0.91–2.07, p_{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.

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Table 3 Association of *KLK14* SNPs and prostate tumour aggressiveness (Gleason score <7 vs. ≥7).

KLK14 SNP	GS <7 (n)	GS ≥7 (n)	OR (95% CI)	p_{trend}
rs73596564	274	685	2.02 (0.22–18.22)	0.533
rs17728459	**264**	**704**	**0.33 (0.15–0.73)**	**0.006**
rs73596556[a]	310	785	–	1.000
rs2691271	316	794	1.00 (0.78–1.28)	0.989
rs12984853	312	801	0.95 (0.79–1.15)	0.621
rs34974522	301	781	0.98 (0.81–1.19)	0.840
rs2691270	300	763	1.18 (0.97–1.43)	0.100
rs2569497	310	786	0.88 (0.70–1.11)	0.280
rs34368430	310	788	1.00 (0.81–1.23)	0.967
rs1880417	319	794	0.50 (0.03–8.06)	0.626
rs4802765	**314**	**791**	**1.31 (1.00–1.72)**	**0.050**
rs7259389	294	756	1.14 (0.87–1.50)	0.343
rs73596529	310	787	1.09 (0.82–1.46)	0.540
rs7260199	302	786	1.04 (0.85–1.28)	0.676
rs12609392	300	785	1.01 (0.84–1.22)	0.905
rs73051038	313	807	1.38 (0.91–2.07)	0.126
rs2072689	259	610	0.84 (0.69–1.03)	0.099
rs2235100	258	602	1.14 (0.87–1.49)	0.359
rs2691244	259	606	1.07 (0.87–1.33)	0.522
rs62114145	300	760	0.85 (0.66–1.09)	0.192
rs62114144	301	783	0.87 (0.68–1.11)	0.255
rs35287116	**312**	**806**	**1.28 (1.06–1.56)**	**0.012**
rs2569491	295	754	0.98 (0.79–1.21)	0.845
rs2569490[a]	296	771	–	1.000
rs10500304	258	603	1.18 (0.95–1.46)	0.130
rs10445581	259	611	1.16 (0.92–1.45)	0.207
rs867192	252	591	1.17 (0.90–1.53)	0.249
rs11671942	260	609	1.08 (0.76–1.52)	0.676
rs11671800	260	606	1.10 (0.84–1.43)	0.509
rs66613646	306	765	1.14 (0.89–1.45)	0.302
rs2569487	299	762	0.89 (0.72–1.09)	0.265
rs2569485	313	805	1.03 (0.85–1.24)	0.768
rs7253072	299	762	0.87 (0.64–1.18)	0.368
rs7253169	311	797	1.13 (0.83–1.55)	0.437
rs2691215	274	685	1.04 (0.85–1.28)	0.716

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.
[a]SNP too infrequent in these groups to calculate OR (95% CI).

Table 4 Association of *KLK14* SNPs and prostate tumour aggressiveness (Gleason score ≤6 vs. ≥8).

KLK14 SNP	GS ≤6 (n)	GS ≥8 (n)	OR (95% CI)	p_{trend}
rs73596564[a]	274	128	–	1.000
rs17728459	264	176	0.15 (0.02–1.15)	0.068
rs73596556[b]	310	151	–	
rs2691271	316	158	0.88 (0.61–1.28)	0.506
rs12984853	312	160	1.05 (0.79–1.41)	0.733
rs34974522	301	151	1.06 (0.79–1.42)	0.720
rs2691270	300	162	1.14 (0.86–1.51)	0.367
rs2569497	310	151	0.85 (0.59–1.21)	0.366
rs34368430	310	152	1.10 (0.80–1.51)	0.571
rs1880417	319	158	–	1.000
rs4802765[a]	314	157	1.18 (0.80–1.74)	0.401
rs7259389	294	161	1.16 (0.79–1.72)	0.447
rs73596529	310	151	1.22 (0.81–1.83)	0.349
rs7260199	302	157	0.77 (0.56–1.05)	0.095
rs12609392	300	157	1.27 (0.95–1.68)	0.103
rs73051038	**313**	**163**	**1.94 (1.10–3.40)**	**0.021**
rs2072689	259	145	0.95 (0.71–1.27)	0.713
rs2235100	258	145	1.30 (0.89–1.88)	0.173
rs2691244	259	144	1.11 (0.82–1.51)	0.504
rs62114145	300	161	0.89 (0.62–1.29)	0.547
rs62114144	301	157	0.84 (0.58–1.22)	0.371
rs35287116	**312**	**162**	**1.38 (1.03–1.85)**	**0.030**
rs2569491	295	162	0.96 (0.70–1.32)	0.798
rs2569490[a]	296	154	–	1.000
rs10500304	258	146	1.24 (0.91–1.68)	0.169
rs10445581	259	145	1.19 (0.86–1.64)	0.294
rs867192	252	142	1.18 (0.82–1.72)	0.377
rs11671942	260	145	1.10 (0.67–1.81)	0.697
rs11671800	260	145	1.10 (0.75–1.61)	0.632
rs66613646	306	147	1.11 (0.76–1.61)	0.591
rs2569487	299	162	0.87 (0.63–1.19)	0.376
rs2569485	313	162	1.08 (0.81–1.44)	0.583
rs7253072	299	162	0.92 (0.59–1.45)	0.725
rs7253169	311	163	1.26 (0.80–1.98)	0.312
rs2691215	274	128	1.11 (0.80–1.53)	0.538

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.
[a]SNP too infrequent in these groups to calculate OR (95% CI).
[b]Rare allele was not detected in either group.

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

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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 examined 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, KLK5 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

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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 U/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 (Invitrogen). Quantitative PCR (qPCR) was performed with ABI PRISM 7000, 7300 and 7900 thermocyclers and SYBR Green PCR Master Mix (Applied Biosystems, Scoresby, Australia). *KLK14*, *GAPDH* and *18S* primer sequences have previously been reported (Lawrence et al., 2010). The comparative C_T ($\Delta\Delta C_T$) method was used to calculate relative gene expression compared with control samples.

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 Biologics (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.apccbioresource.org.au/index.html). Men presented to urologists with lower urinary tract symptoms and/or abnormal serum PSA, and 72% of cases possessed prostate 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), CHIP SNPper (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 genome-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 ($r^2 \geq 0.80$) 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 (r^2<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 (H_2O) 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

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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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