Compartmentalized expression of kallikrein 4 (*KLK4*/hK4) isoforms in prostate cancer: nuclear, cytoplasmic and secreted forms

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Abstract

The prostate-specific antigen-related serine protease gene, kallikrein 4 (*KLK4*), is expressed in the prostate and, more importantly, overexpressed in prostate cancer. Several *KLK4* mRNA splice variants have been reported, but it is still not clear which of these is most relevant to prostate cancer. Here we report that, in addition to the full-length *KLK4* (*KLK4*-254) transcript, the exon 1 deleted *KLK4* transcripts, in particular, the 5'-truncated *KLK4*-205 transcript, is expressed in prostate cancer. Using V5/His6 and green fluorescent protein (GFP) carboxy terminal tagged expression constructs and immunocytochemical approaches, we found that hK4-254 is cytoplasmically localized, while the N-terminal truncated hK4-205 is in the nucleus of transfected PC-3 prostate cancer cells. At the protein level, using anti-hK4 peptide antibodies specific to different regions of hK4-254 (N-terminal and C-terminal), we also demonstrated that endogenous hK4-254 (detected with the N-terminal antibody) is more intensely stained in malignant cells than in benign prostate cells, and is secreted into seminal fluid. In contrast, for the endogenous nuclear-localized N-terminal truncated hK4-205 form, there was less difference in staining intensity between benign and cancer glands. Thus, *KLK4*-254/hK4-254 may have utility as an immunohistochemical marker for prostate cancer. Our studies also indicate that the expression levels of the truncated *KLK4* transcripts, but not *KLK4*-254, are regulated by androgens in LNCaP cells. Thus, these data demonstrate that there are two major isoforms of hK4 (*KLK4*-254/hK4-254 and *KLK4*-205/hK4-205) expressed in prostate cancer with different regulatory and expression profiles that imply both secreted and novel nuclear roles.

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Introduction

Prostate cancer is the most common non-skin malignancy and the second leading cause of cancer death in males in the USA and other Western countries (Jemal *et al.* 2004). Prostate-specific antigen (PSA), also known as human kallikrein 3 (hK3), has been used widely as a biomarker for prostate cancer (Rittenhouse *et al.* 1998, Borgono & Diamandis 2004, Clements *et al.* 2004, Khan *et al.* 2004). The related human kallikrein 2 (hK2) has shown potential as an adjunct biomarker for the discrimination of organ-confined disease (Bangma *et al.* 2004) and lymph-node

metastasis (Shariat *et al.* 2003). The genes encoding these serine proteases belong to a tightly clustered kallikrein gene (*KLK*) (Diamandis *et al.* 2000) locus comprising 15 members on human chromosome 19q13.4 (Gan *et al.* 2000, Harvey *et al.* 2000, Clements *et al.* 2001, Yousef & Diamandis 2001). Many of the *KLKs*/hKs are aberrantly expressed in hormone-dependent cancers (Borgono & Diamandis 2004, Clements *et al.* 2004) and, in addition to PSA and *KLK2*/hK2, *KLK4*/hK4 particularly is of increasing interest in relation to prostate cancer (Nelson *et al.* 1999, Korkmaz *et al.* 2001, Day *et al.* 2002, Obiezu *et al.* 2002, Xi *et al.* 2004).

KLK4/hK4, independently identified by several groups, was variously named prostase, KLK-Like 1, PRSS17, androgen-related message 1/ARM1 and enamel matrix serine protease 1/EMSP1 (Nelson *et al.* 1999, Stephenson *et al.* 1999, Yousef *et al.* 1999, Hu *et al.* 2000, Korkmaz *et al.* 2001). Earlier studies suggested that hK4 protein levels are not elevated in prostate cancer, but quantitative RT-PCR analysis has since indicated that *KLK4* mRNA levels are clearly elevated and associated with cancer progression (Day *et al.* 2002, Obiezu *et al.* 2002). However, alternative mRNA splicing is a common feature in the *KLK* family, and a perplexing aspect of all studies to date is that it is not always clear which splice variants or isoforms of *KLK4*/hK4 were being measured and thus, which are the most relevant to prostate cancer progression.

To date, over 80 variant transcripts have been reported for the 15 members of the *KLK* family (Kurlender *et al.* 2005). Although their full characterization in terms of expression patterns and function remains to be described, studies from our group and others have reported tissue- or cancer-specific mRNA variants for *KLK4*, *KLK5*, *KLK7*, *KLK8* and *KLK11* in malignant prostate and ovarian tissues (Dong *et al.* 2001, 2003, Magklara *et al.* 2001, Nakamura *et al.* 2003, Kurlender *et al.* 2004). Therefore, the mRNA splice variants and protein isoforms of the kallikrein family of serine proteases are emerging as an important group of tumour biomarkers.

The kallikrein 4 gene (*KLK4*) consists of five exons, and several alternative *KLK4* transcripts (reviewed by Kurlender *et al.* 2005) have been reported (Obiezu & Diamandis 2000, Dong *et al.* 2001, Korkmaz *et al.* 2001, Myers & Clements 2001). These include full-length *KLK4* (*KLK4-254*), exon 1 deleted forms that encode proteins missing the signal peptide and pro-region of the zymogen, and several transcripts with splice variations between exons 2 and 5. The latter exhibit a frame shift in the coding region and generate premature stop codons giving rise to truncated protein products that do not have the essential serine and/or aspartic acid residues of the catalytic triad (Obiezu & Diamandis 2000, Dong *et al.* 2001, Korkmaz *et al.* 2001, Myers & Clements 2001). However, two *KLK4* transcripts, the full-length *KLK4-254* and one of the exon 1 deleted transcripts (*KLK4-205*), encode potentially active serine proteases. *KLK4-254* encodes a 254-amino-acid (aa) preproenzyme (hK4-254), while *KLK4-205* encodes an N-terminally truncated 205-amino acid protein (hK4-205). Due to the loss of its signal peptide and pro-region, hK4-205 is unlikely to

be secreted and may be constitutively active. To date, uncertainties abound as to which of these hK4 isoforms are secreted and where they are precisely localized in prostate cells (Day *et al.* 2002, Obiezu *et al.* 2002, Harvey *et al.* 2003, Simmer & Bartlett 2004, Xi *et al.* 2004).

The present study has used a unique suite of three anti-hK4 polyclonal antibodies immunoreactive against the N-terminus (which does not detect the N-terminal truncated proteins derived from exon 1 deleted forms), midregion and C-terminus of hK4 respectively, and has shown that there are two major isoforms of hK4 (*KLK4-254*/hK4-254 and *KLK4-205*/hK4-205) expressed in prostate cancer. Furthermore, we have shown that these two isoforms have a differential cellular localization and therefore probably different functional roles, and that hK4-254 may be a useful immunohistochemical marker for prostate cancer.

Materials and methods

Human cell lines, tissues and cell-culture conditions

Cell lines from the prostate (RWPE1, RWPE2, BPH-1, PC-3 and LNCaP), human salivary gland (HSG), ovarian cancer (OVCAR-3 and PEO4) and breast cancer (T47D) were from the American Type Culture Collection (Manassas, VA, USA), and were grown according to supplied specifications. Neonatal foreskin fibroblast cells (NFF) were a generous gift from Dr Peter Parsons (Queensland Institute of Medical Research), and were cultured as described previously (Qiu *et al.* 2000). Primary cultures of normal ovarian epithelial (NOE) cells were used as described previously (Dong *et al.* 2003). Lymphocytes were from a 30-year-old healthy man with ethics approval. For androgen-regulation studies, LNCaP cells were cultured in 2% (v/v) charcoal-stripped fetal calf serum (FCS) for 48 h and then treated with the synthetic androgen R1881 (10 nm in ethanol) or 0.1% (v/v) ethanol vehicle for a further 24 h. Experiments were repeated three times with pooled cells (in triplicate). Twelve tissues from benign prostatic hyperplasia (BPH) ($n = 6$) and prostate cancer (PCa) ($n = 6$), two ejaculate samples and archival paraffin-wax-embedded prostate tissue blocks from eight patients were obtained, with appropriate institutional ethics committee approval and informed consent from the patients. These patients, 50–60 years old, underwent transurethral resection of the prostate (TURP) or radical prostatectomy at Royal Brisbane Hospital. The Gleason scores for prostate cancer

tissues ranged from 3+4 to 4+5. Two ejaculate samples were from patients negative and positive respectively for malignancy (Gleason 3+4) by transrectal ultrasound biopsy. The ejaculate samples were collected into Hanks' balanced salt solution (Sigma-Aldrich, Castle Hill, NSW, Australia) and centrifuged, and the supernatant (diluted seminal fluid) was stored at -80°C for further analysis.

Reverse transcription–PCR (RT–PCR) and sequencing analysis

Total RNA was isolated from tumour cells or tissues using TRIzol reagent (Invitrogen), treated with RNase-free DNase I (Invitrogen) and reverse-transcribed with random hexamers pd(N)₆ (Roche) and Superscript II (Invitrogen). PCR with *KLK4*-specific primers K4Ex2S and K4Ex5AS (Dong *et al.* 2001), or K4Ex1ATGS (5'-ATGGCCACAGCAGGAAATCCC-3') and K4Ex4AS (5'-CACGCACTGCAGCACGGTAG-3'), was performed with 1 µl cDNA, 0.5 units platinum Taq DNA polymerase (Invitrogen) and 35 cycles with annealing temperatures of 60–62°C. PCR for the internal control, β 2-microglobulin (β 2M) (Dong *et al.* 2003) was carried out with an annealing temperature of 56°C. The PCR products were electrophoresed on a 1.5% (w/v) agarose gel and visualized by ethidium bromide staining. PCR products were gel purified (Qiagen), sequenced at the Australian Genome Research Facility (University of Queensland, Brisbane, Australia), and the DNA sequences were analysed with tBLASTN.

Detection of *KLK4* mRNA variants by quantitative RT–PCR (qRT–PCR)

qRT–PCR reactions with *KLK4* (K4Ex1QS 5'-TACCTCATCTCTTGGTGTCGCA-3' and K4Ex2QAS 5'-ACGCCCCGAGCAGAACAATT-3', K4Ex2QS 5'-GGCACTGGTCATGGAAAACGA-3' and K4Ex3QAS 5'-TCAAGACTGTGCAGGCCAGCC-3', Ex4QS 5'-GAGGGCAAGACCAGAAGGACT-3' and Ex5QAS 5'-TTTCCGAAAGACACAAGGCC-3') or 18S-specific primers (McCulloch *et al.* 2004) were performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR green according to the manufacturer's instructions. 18S ribosomal RNA expression was measured to normalize *KLK4* expression for sample-to-sample differences in RNA input, RNA quality and reverse-transcription efficiency. Each primer set was validated for use in quantitation by the 'CT-values' direct comparison method according to the manufacturer's recommendations (McCulloch *et al.* 2004).

Statistical analysis for qRT–PCR data

The significance of differences between mean values of *KLK4* expression by qRT–PCR of BPH versus PCa was evaluated with the independent two-tailed *t*-test. Differences were considered significant at $P < 0.05$.

In vitro transcription/translation

cDNAs encoding full-length *KLK4-254*, *KLK4-205* and *KLK4-110* with transcript-specific primers with the T7 promoter sequence at the 5'-end were amplified from mRNA extracted from prostate cancer tissue and then cloned into the pGEMT-easy vector (Promega). Reactions were performed with a TNT T7 coupled reticulocyte lysate system (Promega) in the presence of ³⁵S-methionine. The translated product was separated by 12% (w/v) SDS–PAGE, and the signals were visualized by exposure to radiographic film.

Transient expression of full-length hK4-254 and hK4-205 tagged with GFP and V5/His epitope

cDNA encoding full-length hK4-254 and hK4-205 was amplified and subcloned into the pEGFP-N3 vector (C-terminal tagged, BD Biosciences/Clontech, Palo Alto, CA, USA) or the pcDNA3.1V5/His vector (C-terminal tagged, Invitrogen) at the XhoI and BamHI sites. Plasmid DNA was transfected into PC-3 cells with Lipofectamine 2000 (Invitrogen).

Western blot analysis

Cytoplasmic and nuclear proteins were extracted from LNCaP cells with the NE-PER kit (Pierce, Rockford, IL, USA). These extracts (30 µg cytoplasmic; 10 µg nuclear) and ~50 µg seminal fluid protein were separated by 12% SDS–PAGE and transferred to a Protran membrane (Schleicher and Schuell, Dassel, Germany). After blocking with 5% (w/v) skim milk in TBS-Tween 20 (Tris 50 mM, NaCl 30 mM and 0.05% Tween 20), the membrane was incubated with anti-V5 antibody (Invitrogen) (1/2000 dilution in 5% skim milk in TBS-Tween 20), which detects the 15-amino acid V5 epitope tag at the C-terminus of the transiently expressed hK4 proteins, or specific anti-hK4 peptide antibodies (1/500 dilution in blocking solution as above) (Harvey *et al.* 2003) overnight at 4°C. Anti-mouse and anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase (Pierce) (1/2000 in blocking solution) were used, and the signals were visualized on radiographic film by Femto enhanced chemiluminescence (Pierce). Western blot with an anti-PSA polyclonal antibody (DakoCytomation, Botany, Australia) (1/5000 dilution

Figure 1 Expression of *KLK4* mRNA transcripts in different cell lines and prostate tissue RNA. (A) Schematic structures of all reported potential *KLK4* mRNA transcripts (*KLK4-254*, *KLK4-205*, *KLK4-146/195*, *KLK4-110/159* and *KLK4-75*), including the start codon ATG, five exons and introns I–IV, as indicated to the right. Arrowheads indicate the position of the stop codon for each

as above) was used as a positive control for R1881 treatment of LNCaP cells. Anti- β tubulin (Upstate, Charlottesville, VA, USA) (1/3000 dilution in blocking solution) and anti-histone (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1/200 dilution as above) antibodies were used as controls for cytoplasmic and nuclear fractions respectively.

Deglycosylation

Deglycosylation of recombinant hK4-254 and hK4-205 from transiently transfected PC-3 cells and cytoplasmic and nuclear proteins from LNCaP cells was performed with N-glycosidase F (PNGase F, New England BioLabs, Beverly, MA, USA) followed by western blot analysis.

Direct and indirect immunofluorescence (IF) microscopy on cultured cells

PC-3 cells were grown in Lab-Tek II Chamber Slides (Nalge Nunc, Rochester, NY, USA) overnight and then transfected with the GFP constructs. After 24 h, the cells were washed and fixed with 4% (w/v) paraformaldehyde, and the slides were mounted with ImmunoFluor mounting medium (ICN, Aurora, OH, USA). The pcDNA3.1V5/His construct-transfected PC3 cells were fixed with 100% (v/v) methanol, blocked with 2% (w/v) BSA and 0.1% (w/v) saponin in PBS, and then incubated with anti-V5 antibody (Invitrogen) (1/2000 dilution in 0.01% (w/v) saponin in PBS) overnight at 4°C. After washing, cells were incubated with the AlexaFluor 488 goat anti-mouse IgG antibody (1/500 dilution as above) (Molecular Probes, Eugene, OR, USA), and slides were mounted as described above. All cells were examined under a Leitz Fluorescent Compound UV microscope (Leica Microsystems, Gladesville, Australia), and images were acquired with a Nikon DXM1200 digital camera (Coherent Life Sciences, Hilton, Australia). The cultured prostatic cell lines, RWPE1, RWPE2, BPH-1, PC-3 and LNCaP, and

R1881-treated LNCaP cells were examined by indirect IF with anti-hK4-N or anti-hK4-C antibodies (1/200 dilution as above). The staining was visualized by confocal microscopy (Leica) with propidium iodide (50 μ g/ml) counterstaining for nuclear localization.

Immunohistochemistry (IHC) and IF on prostate tissues

Archival paraffin-wax-embedded blocks from two benign and six cancerous prostate tissues with different Gleason scores (3+4 to 4+5) were sectioned (4 μ m), deparaffinized and rehydrated. After antigen retrieval in 5% (w/v) urea in 0.1 M Tris buffer (pH 9.5) and 3% (v/v) H₂O₂ treatment to block endogenous peroxidase, the sections were blocked with normal goat serum (Santa Cruz) and then incubated overnight with anti-hK4-N, anti-hK4-M or anti-hK4-C (each 1/200 dilution in 10% normal goat serum) at 4°C. For IHC staining, the EnVision peroxidase (anti-rabbit) polymer detection system (Dako) was used with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) as the chromogen. The sections were counterstained with Gill's haematoxylin. Ten per cent normal goat serum instead of primary antibody was used for negative controls. Indirect IF and confocal microscopy were also performed as described above.

Results

KLK4 and its mRNA variants are highly expressed by prostate cancer cell lines and prostate cancer tissues

The expression of *KLK4* transcripts in different cell lines was examined by RT-PCR using *KLK4*-specific primers that amplify regions representative of the different transcripts. As in the schematic structure shown in Fig. 1A, RT-PCR with exons 1–4 primers (K4Ex1ATGS and K4Ex4AS) (Fig. 1A) would amplify three potential transcripts: full-length *KLK4-254*

transcript. The dotted-line exons indicate the potential existence of these exons. The transcript lengths produced and exons covered (arrowed lines) by each primer set for RT-PCR and qRT-PCR are shown, with (arrowed lines) the transcripts detected and the expected size indicated. (B) RT-PCR using primers from different exons to amplify regions (a) exons 1–4, (b) exons 2–5 and (c) β 2-microglobulin (β 2M) as the internal control for the integrity of the RNA. Lanes 1–2, primary cultured neonatal foreskin fibroblast cells (NFF) and normal ovarian epithelial cells (NOE); lanes 2–3, ovarian cancer cell lines OVCAR-3 and PEO4; lane 5, lymphocytes; lanes 6–7, prostate cell lines RWPE1 and RWPE2; lane 8, BPH tissue BPH-a; lane 9, PCa tissue PCa-a; lane 10, BPH tissue BPH-b; lane 11, PCa tissue PCa-b; lane 12, prostate cancer cell line PC-3; lane 13, human salivary gland (HSG); lane 14, prostate cancer cell line LNCaP; lane 15, breast cancer cell line T47D; lane 16, no cDNA as negative control. (C) *KLK4* expression levels in the prostate cell lines (RWPE-1, RWPE-2, PC-3 and LNCaP) and BPH ($n=6$) and prostate cancer ($n=6$) tissues with qRT-PCR with primers from exons 1–2, 2–3 and 4–5. Dots represent the individual samples, and bars represent the mean for that data set. Statistical analysis of *KLK4* expression between BPH and PCa, with six patients in each group, was with the levels of *KLK4-254* (exons 1–2, $*P>0.05$), total *KLK4* transcripts (exons 2–3, $**P<0.0001$), and *KLK4-254*, *KLK4-205*, *KLK4-146* and *KLK4-75* transcripts (exons 4–5, $***P<0.002$). Each experiment was repeated 4–5 times. Note the log scale of the lower half of y-axis.

(510 bp); *KLK4-195* (593 bp), which has an additional 83 bp sequence insertion from intron III and would potentially encode a C-terminal truncated protein of 195 aa; and *KLK4-75* (522 bp), which has a 12 bp sequence insertion from intron II and would encode a 75 aa protein. As seen in Fig. 1B (panel (a), exons 1–4), the *KLK4-254* transcript (510 bp product) was amplified predominantly in prostate cell lines (RWPE1, RWPE2, PC-3 and LNCaP), benign (BPH-a and BPH-b) and malignant (PCa-a and PCa-b) prostate tissue samples and human salivary gland (HSG) cells, compared with little or no amplification from human foreskin fibroblast (NFF) cells, lymphocytes, normal ovarian epithelial (NOE) cells, the ovarian cancer cell lines OVCAR-3 and PEO4, and the breast cancer cell line T47D. Some amplification of the *KLK4-75* transcript may also have occurred, as it is difficult to distinguish this 522 bp product from that of the 510 bp *KLK4-254* product. However, we have not observed a protein of the size (75 aa; ~10 kDa) encoded by the *KLK4-75* transcript on Western blot analysis with our N-terminal directed antibody (anti-hK4-N) (Fig. 2A); therefore, we do not believe that this transcript is expressed to any great degree, if at all. No amplification of the 593 bp *KLK4-146* transcript occurred, so we believe that the amplification product seen here with exons 1–4 primers is representative only of the full-length *KLK4-254* transcript (Fig. 1B, panel (a)). Of interest, this amplicon was not as intense as that seen with the exons 2–5 primers, although the same cycle number was used, suggesting a lower level of *KLK4-254* expression than the exon 1 deleted transcripts (Fig. 1B, panels (a) and (b)).

RT-PCR with the exons 2–5 primers (K4Ex2S and K4Ex5AS) (Fig. 1A) was used to amplify all *KLK4* transcripts (Fig. 1A) (Obiezu & Diamandis 2000, Dong et al. 2001, Korkmaz et al. 2001, Myers & Clements 2001). While the *KLK4-254* and exon 1 deleted *KLK4-205* transcripts would give the same PCR product of 526 bp (Obiezu & Diamandis 2000, Dong et al. 2001, Korkmaz et al. 2001, Myers & Clements 2001), four other transcripts could potentially be amplified. These are exon 1 deleted versions of *KLK4-195* and *KLK4-159* (609 and 389 bp) with an intron III insertion or exon 4 deletion that would encode hK4 proteins of 146 and 110 aa respectively, and thus are designated *KLK4-146* and *KLK4-110* (Obiezu & Diamandis 2000, Dong et al. 2001, Korkmaz et al. 2001, Myers & Clements 2001). *KLK4-75* and its exon 4 deleted forms would give PCR products of 538 and 401 bp respectively. As seen in Fig. 1B, multiple PCR products indicative of the above transcripts were amplified, although the 526 bp product was the most intense, suggesting that the *KLK4-254* and *KLK4-205* transcripts are the

predominant transcripts expressed in the prostate. Of interest, and in sharp contrast to expression of *KLK4-254*, these exon 1 deleted transcripts were also expressed in non-prostatic tissues (Fig. 1B, panels (a) and (b)). PCR for β 2-microglobulin (β 2M), used as the internal control (Fig. 1B, panel (c)), showed a consistent pattern of expression in all samples, indicating the integrity of the RNA.

For further determination of the expression levels of the two major *KLK4* mRNA transcripts (*KLK4-254* and *KLK4-205*) in prostate cell lines and tissues, three sets of qRT-PCR primers were designed to amplify the *KLK4-254* transcript alone (exons 1–2) and the *KLK4-254* and *KLK4-205* transcripts together (exons 2–3 and 4–5), as described above. Clear amplification was achieved with the three primer sets, although the log scale amplifying phase for the exons 1–2 product appeared eight cycles later than the exons 2–3 and 4–5 products (data not shown) indicating that the combined levels of *KLK4-254* and *KLK4-205* are significantly higher than those of full-length *KLK4-254*. Surprisingly, the amplification levels obtained with the exons 2–3 and 4–5 primers were not the same, presumably indicating some contribution from the exon 4 deleted transcript (*KLK4-110*) in the exons 2–3 PCR. Thus, from the combined RT-PCR and qRT-PCR analyses, we would suggest that the exons 4–5 amplification primarily represents *KLK4-205* (Fig. 1C), as there was a very low level of *KLK4-146* (Fig. 1B, panel (b)). The normalized qRT-PCR values clearly show that *KLK4-205*, in particular, is higher in both LNCaP and PC-3 cancer cell lines than in RWPE1 and RWPE2 prostate cells (in Fig. 1C, note the log scale of bottom half of y-axis). In addition, *KLK4-205* transcripts (exons 4–5 amplification) were significantly higher in PCa ($n=6$) than BPH ($n=6$) tissues ($**P=0.002$, Fig. 2C), as were the combined *KLK4-205/KLK4-110* levels (exons 2–3 amplification) ($**P=0.0001$, Fig. 1C). The *KLK4-254* transcript levels (exons 1–2 amplification) also appeared higher in PCa than BPH tissues, although they did not reach significance ($*P=0.09$, Fig. 1C).

***KLK4-254* and *KLK4-205* can be translated, and hK4-254 is glycosylated and secreted into seminal fluid**

To determine hK4 expression at the protein level, three affinity-purified antibodies derived from N-terminal, midregion and C-terminal regions of hK4-254 were produced and designated anti-hK4-N, anti-hK4-M and anti-hK4-C respectively (Fig. 2A) (Harvey et al. 2003). Theoretically, given the RT-PCR analysis

Figure 2 Protein expression of hK4-254 and hK4-205. (A) The potential translated proteins from the *KLK4* transcripts, hK4-254, hK4-205, hK4-146, hK4-110 and hK4-75, and the alignment of the anti-hK4 peptide antibodies used in this study. Histidine (His) aspartic acid (Asp) and serine (Ser) of the catalytic triad are indicated. The N-glycosylation sites are indicated by Asn (asparagine) in hK4-254 and hK4-205. (B) Comparison of hK4-254, hK4-205 and hK4-110 *in vitro* transcription-translation detected by ³⁵S-labelling. Lane 1, control reaction from a luciferase cDNA giving a protein product of the predicted size (61 kDa); lanes 2–5, transcription/translation from PCR products containing nucleotides of full-length *KLK4-254*, *KLK4-205*, *KLK4-110* and no DNA respectively; lanes 6–9, Western blot analysis with anti-V5 antibody for pre- and post-deglycosylation of recombinant hK4-254 and hK4-205; lanes 6-7, hK4-254 without (–) or with (+) PNGase F; lanes 8–9, hK4-205 without (–) or with (+) PNGase F. (C) Western blot analysis with anti-hK4 antibodies pre- and post-deglycosylation. Lanes 1–6, anti-hK4-N antibody detection of recombinant hK4-254, endogenous cytoplasmic and nuclear hK4 from LNCaP cells without (–) or with (+) PNGase F; lanes 7–10, anti-hK4-C antibody detection of endogenous hK4 from LNCaP cells. (D) Western blot analysis with anti-hK4-N on seminal fluid samples from a non-PCa (–) patient and a PCa (+) patient.

above, the anti-hK4-N should detect only full-length hK4-254 and hK4-75 (if present) and the anti-hK4-M will detect all hK4 isoforms except for the hK4-75 isoform, while the anti-hK4-C should detect the hK4-254 and hK4-205 forms only, as all other isoforms have C-terminal truncations prior to this sequence (Fig. 2A).

To determine whether the two major *KLK4* transcripts were translated, we used the TNT T7 coupled reticulocyte lysate system. ³⁵S-methionine-labelled, translated protein products of 32 and 29 kDa were detected from the *KLK4-254* and *KLK4-205* constructs respectively (Fig. 2B), with the luciferase-positive control translating a 70 kDa protein as expected and the no-cDNA control appropriately negative (Fig. 2B). Given the potential contribution of the exon 4 deleted transcript, *KLK4-110*, to the overall expression levels seen above, we also examined whether this transcript could be translated; however, no ³⁵S-methionine-labelled product was observed.

The translational ability of *KLK4-254* and *KLK4-205* cDNA to produce protein in cultured cells was confirmed by transient transfection of the respective expression constructs into PC-3 prostate cancer cells, followed by western blot analysis with an anti-V5 antibody (Fig. 2B). The size of the expressed hK4-205

(29 kDa) was consistent with that observed for the ³⁵S-methionine-labelled, *in vitro* translated protein product. The expressed hK4-254, as detected by either the anti-V5 or anti-hK4-N antibodies, was ~35 kDa (Fig. 2B and C), and was slightly larger than that seen in the *in vitro* translation system but consistent with the size detected in our previous transient transfections in COS-7 cells (Harvey *et al.* 2003). The endogenous intracellular hK4 detected in LNCaP cell extracts with either the anti-hK4-N or anti-hK4-C was of a different size (~45 kDa) from that of transiently transfected hK4-254 or hK4-205 (Fig. 2C). Additional bands (~60 and 90 kDa) were also detected with anti-hK4-N. This presumably reflects other post-translational modifications or complexed forms of hK4. Of interest, some of these bands (particularly the 45 kDa band) were

also seen in the transiently transfected hK4-254 PC-3 cells. However, the anti-hK4-N antibody detected a ~32 kDa protein in the seminal fluid of two men, one without prostate cancer (PCa(-)) and the other with prostate cancer (PCa(+)) by TRUS (Fig. 2D), suggesting that the secreted hK4-254 is now uncomplexed and probably active hK4. Additional bands at ~45 and 42 kDa were observed in the seminal fluid sample from the cancer patient only, suggesting that these bands may be more cancer specific, but their identity is not known at this time. This latter protein expression result is consistent with our qRT-PCR results and emphasizes that *KLK4-254/hK4-254* is indeed expressed in, and secreted from, the prostate of prostate cancer patients.

We also assessed whether the recombinant and endogenous forms of these two proteins are glycosylated, as there is a putative N-glycosylation site in hK4-254 (Asn 169) and hK4-205 (Asn 120, Fig. 2A). On treatment with PNGase F, the transiently transfected hK4-254 band (~35 kDa) shifted to a lower molecular mass (~33 kDa) as detected by anti-V5 (Fig. 2B) and anti-hK4-N antibodies (Fig. 2C), but there was no change in the size of the recombinant hK5-205 (~29 kDa) as detected by the anti-V5 antibody (Fig. 2B). Similarly, there was no size alteration after PNGase F treatment of either endogenous cytoplasmic or nuclear hK4 proteins extracted from LNCaP cells (as detected by either the anti-hK4-N or anti-hK4-C antibodies) (Fig. 2C). This indicates that while the recombinant hK4-254 is glycosylated neither the endogenous LNCaP hK4-254 nor -205 protein is glycosylated.

hK4-254 is predominantly localized in the cytoplasm, but hK4-205 is in the nucleus

Of particular note was that hK4 immunoreactive material was detected in both cytoplasmic and nuclear extracts from LNCaP cells (Fig. 2C). To explore further the cellular localization of hK4 variants, transient transfection of recombinant *KLK4-254* and *KLK4-205* constructs in pEGFP-N3 with C-terminal-tagged GFP (Fig. 3A–C) and pcDNA3.1V5/His with a C-terminal-tagged V5/His epitope (Fig. 3D–F) was performed in PC-3 cells. The hK4-254 protein was essentially cytoplasmically localized (Fig. 3A and D), but hK4-205 was predominantly localized to the nucleus (Fig. 3B and E). A diffuse fluorescence pattern was observed in both the nucleus and cytoplasm with the control GFP vector transfection (Fig. 3C), and no IF staining was observed on pcDNA3.1V5/His vector transfection, as expected (Fig. 3F). To determine the endogenous expression pattern, IF and confocal microscopy were performed on

cultured RWPE1 (Fig. 3G and K), RWPE2 (Fig. 3H and L), BPH-1 (Fig. 3I and M) and LNCaP (Fig. 3J and N) cells with the anti-hK4-N and anti-hK4-C antibodies. hK4 protein was primarily localized cytoplasmically by the anti-hK4-N antibody (Fig. 3G–J) in all cells examined, consistent with transient expression of the full-length hK4-254 in PC-3 cells (Fig. 3A and D). However, a predominant nuclear localization was observed with anti-hK4-C (Fig. 3K–N) similar to that observed after transfection of the *KLK4-205* constructs (Fig. 3B and E). These results indicate that the endogenous hK4 detected by anti-hK4-N is hK4-254 while the anti-hK4-C detects primarily hK4-205.

Androgen regulation in LNCaP cells

Given the discrepancy in the literature with respect to the fold changes (1.8–20-fold) observed in *KLK4/hK4* expression after androgen treatment (Nelson *et al.* 1999, Stephenson *et al.* 1999, Yousef *et al.* 1999, Hu *et al.* 2000, Korkmaz *et al.* 2001), we wished to determine which isoform was androgen regulated in LNCaP cells. After R1881 treatment for 24 h, there was a ~2–4-fold increase in the level of total *KLK4* transcripts as detected by qRT-PCR with exons 2–3 ($*P=0.019$) (Fig. 4A) or exons 4–5 ($**P=0.0013$) primers, while no increase was observed with exons 1–2 primers (Fig. 4A). Of interest, and similar to that observed with our earlier qRT-PCR (Fig. 1C), the level observed with the exons 2–3 PCR was greater than that seen with the exons 4–5 primers. A significant upregulation in PSA expression was observed as expected (~10.5-fold, $**P=0.0001$) (Fig. 4A). At the protein level, there appeared a small increase in the band intensity of nuclear protein as detected by anti-hK4-N in R1881-treated cells versus the control cells (Fig. 4C). However, no increase of nuclear or cytoplasmic hK4, or change in localization was observed on IHC and IF analyses in androgen-treated cells (data not shown). In addition, there was no change in band intensity of the nuclear fraction by anti-hK4-M or anti-hK4-C, or in cytoplasmic protein as detected by all three anti-hK4 antibodies (Fig. 4C), while PSA protein levels were dramatically increased (Fig. 4D). These results indicate that *KLK4/hK4* is minimally upregulated by R1881 treatment at either the mRNA or protein levels. A ~45 kDa protein band was consistently detected in the cytoplasmic extracts by the three anti-hK4 peptide antibodies in addition to a 32 kDa band by the anti-hK4-M antibody and a ~80 kDa band by the anti-hK4-C antibody respectively (Fig. 4C). Like that seen in Fig. 2C, several other bands were also observed with the anti-hK4-N antibody. In the nuclear extracts, the ~45 kDa proteins

Figure 3 Expression of hK4 isoforms in cultured cells. Fluorescence microscopy of transient transfection of *KLK4-254*-GFP-N3 (A), *KLK4-205*-GFP-N3 (B) and GFP-N3 (C) constructs in PC-3 cells. Immunofluorescence (IF) microscopy with an anti-V5 antibody on transient transfection of *KLK4-254*-pcDNA3.1V5/His (D), *KLK4-205*-pcDNA3.1V5/His (E) and pcDNA3.1V5/His (F) in PC-3 cells. IF staining with anti-hK4-N (G–J) and anti-hK4-C (K–N) antibodies and confocal microscopy on cultured RWPE1 (G and K), RWPE2 (H and L), BPH-1 (I and M) and LNCaP cells (J and N).

were the predominant forms detected by all three antibodies, with two additional ~52 and ~60 kDa bands detected by the anti-hK4-M antibody (Fig. 4C). Surprisingly, there appeared to be three nuclear isoforms of ~45–50 kDa. Western blotting with anti-tubulin and histone antibodies showed no contamination between the cytoplasmic and nuclear protein extracts (Fig. 4D).

Endogenous hK4-254 and hK4-205 are highly expressed in prostate cancer but with different cellular localization

To determine the expression of endogenous hK4-254 and hK4-205 in prostate tissues, IHC was performed

with anti-hK4-N (Fig. 5A, B, and D–F), anti-hK4-M (Fig. 5G) and anti-hK4-C (Fig. 5C, H and I) antibodies. With the anti-hK4-N, there was essentially no staining observed in normal prostate (Fig. 5A) and benign prostatic hyperplasia (BPH) (Fig. 5B) tissues. Nuclear staining was observed in normal/prostatic intraepithelial neoplasia (PIN) tissues (Fig. 5C) with anti-hK4-C antibody. For the prostate cancer tissue samples, there was little staining in benign glands (BNG) (Fig. 5D and E) for either secretory (Fig. 5E, open arrows) or basal cells (Fig. 5E, arrowheads), but strong staining was present in the prostate cancer cells (Ca.) (Fig. 5D and E, arrows). In the majority of sections, anti-hK4-N staining was predominantly found in the cytoplasm of prostate cancer cells

Figure 4 Regulation of *KLK4*/hK4 and PSA at mRNA and protein levels by R1881 in LNCaP cells. (A) qRT-PCR analysis of *KLK4* expression in R1881-treated LNCaP cells with *KLK4* exons 1–2, 2–3, 4–5 or PSA-specific primers. Student's *t*-test was used for statistical analysis of R1881 regulation. Exons 1–2, no significance; exons 2–3, $*P = 0.019$; exons 4–5, $**P = 0.0013$; and PSA, $**P = 0.0001$. (B) The regions detected by the anti-hK4 peptide antibodies (anti-hK4-N, anti-hK4-M and anti-hK4-C) used in this study. (C) Western blot analysis with anti-hK4-N, anti-hK4-M and anti-hK4-C antibodies on endogenous cytoplasmic (Cyto) and nuclear (Nu) hK4 proteins from LNCaP cells without (–) or with (+) R1881 treatment respectively. (D) An anti-PSA antibody was used as a positive control for R1881 treatment; tubulin and histone antibodies were used as controls to confirm that there was no cross-contamination of cytoplasmic and nuclear fractions respectively.

(Fig. 5E, arrows), but, occasionally, positive staining was found in both the cytoplasm (Fig. 5F, open arrows) and nucleus (Fig. 5F, arrows) of the cancer cells. To distinguish the expression of endogenous hK4-254 from the possible expression of the hK4-75 isoform (Fig. 2A) (Obiezu & Diamandis 2000), IHC with the anti-hK4-M (which would not detect hK4-75) revealed a strong cytoplasmic staining of the cancer cells (Fig. 5G, arrows) compared with no staining of

BNG glands (Fig. 5G, open arrow), suggesting that both anti-hK4-N and anti-hK4-M antibodies detect the endogenous hK4-254 (Harvey *et al.* 2003). In contrast, mainly nuclear staining was found by the anti-hK4-C antibody (Fig. 5H and I, arrows), with often a stronger signal in cancer cells than in BNG glands (Fig. 5I, arrows). No stromal cells were stained with any of the antibodies. No IHC staining was observed for the negative control when the primary

Figure 5 Representative immunohistochemical (IHC) staining patterns of endogenous hK4 protein expression in prostate tissue sections ($n = 8$) using anti-hK4-N (A, B and D–F), anti-hK4-M (G) and anti-hK4-C (C, H and I) antibodies. Both normal prostate glands (A) and benign prostatic hyperplasia (BPH) (B) show little staining. (C) Normal or prostatic intraepithelial neoplasia (PIN) with nuclear staining. (D) Low magnification ($\times 40$) of a prostate cancer with strong staining in cancer cells (Ca.), but not in benign glands (BNG). (E) Higher magnification ($\times 120$) of an area from (D) showing a lack of staining of secretory (open arrows) or basal prostate cells (arrowheads) in BNG but strong staining in the cytoplasm of cancer cells (Ca., arrows). The hK4 staining was mainly present in the cytoplasm of the secretory prostatic cancer cells (arrows). (F) The positive staining of both cytoplasm (open arrows) and nucleus (arrows) of all cancer cells of a prostate cancer section. (G) Similar staining pattern also observed with anti-hK4-M antibody: strong cytoplasmic staining in cancer cells (Ca., arrows) but no staining in BNG glands (open arrow). (H) With anti-hK4-C, most of the secretory cells show intense positive staining in the nucleus (arrows) but no stromal cells are positively stained (open arrows). (I) Intense positive nuclear staining of cancer cells (arrows) with anti-hK4-C compared with the BNG glands. (J) IHC staining without primary antibody as the negative control. IF staining and confocal microscopy confirmed cytoplasmic staining with anti-hK4-N (K) and nuclear staining with anti-hK4-C (L).

antibody was omitted (Fig. 5J). The IHC result was also confirmed by IF staining and confocal microscopy with cytoplasmic staining detected with anti-hK4-N (Fig. 5K, arrows) and nuclear staining

with anti-hK4-C (Fig. 5L, arrows) in prostate tissue sections. Stacked confocal images also confirmed the nuclear, as opposed to nuclear membrane, localization of hK4 (data not shown).

Discussion

In this study, we have provided further evidence regarding the major *KLK4* transcripts expressed in benign and malignant prostate tissues, the size of recombinant and endogenous hK4 protein isoforms and their cellular localization. Our data indicate that there are two major endogenous isoforms of hK4 expressed in the prostate. Significantly, and contrary to previous reports (Day *et al.* 2002, Obiezu *et al.* 2002, Xi *et al.* 2004), we have demonstrated that the *KLK4-254* transcript that encodes the classical kallikrein serine protease, prepro-hK4-254, and the hK4-254 protein are overexpressed in prostate cancer. Consistent with the presence of its secreted form in seminal fluid, the hK4-254 protein is also predominantly localized in the cytoplasm, indicating that hK4-254 probably has a classical serine protease role in cancer progression. Although higher expression levels were observed for the exon 1 deleted *KLK4-205* transcript, as previously reported (Day *et al.* 2002, Obiezu *et al.* 2002, Xi *et al.* 2004), less discrimination was seen at the protein level between benign and malignant glands. This novel N-terminally truncated hK4-205 form, which does not have a signal peptide or pro-region, was localized in the nucleus as previously reported (Xi *et al.* 2004), supporting a novel nuclear role for this isoform.

We have demonstrated that the full-length *KLK4-254* transcript is indeed present in prostate tissues. The lower level of the exon 1 containing transcripts could be the reason for the failure to identify this exon with 5'-RACE (Nelson *et al.* 1999, Korkmaz *et al.* 2001), as exon 2, which contains the transcript start site for the exon 1 deleted forms, is more easily amplified by such a technique. The combined RT-PCR and qRT-PCR data presented here do show that *KLK4-254* and *KLK4-205* are the major transcripts in the prostate, and the exon 1 deleted *KLK4-205* transcript is more highly expressed. This finding is consistent with previous reports in which the primers used would amplify a common region (exons 2–3) of all *KLK4* transcripts (Day *et al.* 2002, Obiezu *et al.* 2002, Kurlender *et al.* 2005), but the implication was that the exon 1 deleted forms were the most prevalent. Given the lower levels of *KLK4-254* detected here, this is indeed true.

In our hands, both full-length *KLK4-254* and *KLK4-205* transcripts, but not *KLK4-110*, can be *in vitro* translated, and only the recombinant hK4-254 is N-glycosylated, although both hK4-254 and hK4-205 have N-glycosylation sites. As with hK4-205, no N-glycosylation of the endogenous

LNCaP cell cytoplasmic or nuclear hK4 was detected on Western blot analysis, suggesting that the intracellular endogenous hK4 in LNCaP cells may be the hK4-205 isoform, but this is yet to be formally confirmed. Indeed, since all three antibodies detect similar bands, with some minor exceptions (Fig. 4B), it is not yet clear which of the endogenous intracellular forms in LNCaP cells represent hK4-254, hK4-205 or other isoforms, although there are clearly different post-translation modifications.

Our western blot analysis does support the notion that hK4-254 is expressed in the prostate, and secreted, in addition to N-terminally truncated forms. The anti-hK4-N antibody, raised from an N-terminal region of the active hK4-254, detected both the secreted endogenous hK4 at a molecular mass of 30–32 kDa in seminal fluid samples from both benign and cancer patients and the recombinant hK4-254 at 34 kDa. Of interest, in seminal fluid, the 45 kDa band detected by the anti-hK4-N was similar to the endogenous hK4 seen in the cytoplasmic and nuclear fractions from LNCaP cells. A likely explanation is that this and other higher molecular mass bands are bound forms of hK4 possibly complexed with inhibitors, as has been reported for other kallikreins (Mikolajczyk *et al.* 1999, Chao *et al.* 2001). Of interest, and perhaps surprisingly, the anti-hK4-N antibody also detected a small amount of endogenous hK4 (presumably hK4-254) in the nucleus by IHC and western blot (Figs 4C and 5F). This may reflect translocation of some hK4-254, as well as hK4-205, into the nucleus.

The 45 kDa protein identified was the predominant intracellular hK4 isoform, as has been previously reported for a serpin, maspin (Sood *et al.* 2002). The triplet of 45 kDa proteins in the nucleus is striking and presumably reflects further post-translational modifications and/or different isoforms of hK4. The identity of the additional bands (at ~30 kDa in the cytoplasm and ~52 and 60 kDa in the nucleus) detected by anti-hK4-M is not clear. Although we cannot discount that any of these bands may represent isoforms of the less highly expressed *KLK4-146* or *KLK4-110* transcripts, this is less likely given our *in vitro* translation data. Similarly, any hK4-75 protein, if translated, would not be detected by this antibody. In addition, we did not see any bands at <20 kDa that would represent unmodified forms of these proteins with any of the antibodies. Moreover, since the C-terminal antibody will recognize only the hK4-254 and hK4-205 forms and the major proteins (~45 kDa) detected were similar for all three antibodies, we believe that these bands are representative of the hK4-254 or hK4-205 isoforms only.

A most striking observation, however, was that, on IHC staining, these antibodies detected a differential localization of endogenous hK4, in which the anti-hK4-N and anti-hK4-M detected predominantly the cytoplasmic form, but the anti-hK4-C showed a clear nuclear staining in cultured prostate cells and prostate cancer tissue sections. The cytoplasmic and apical localization of hK4 in prostate epithelial cells from tissue sections has been previously reported with an antibody raised against recombinant hK4-254 (Obiezu *et al.* 2002), consistent with our results with the anti-hK4-N and anti-hK4-M antibodies that detect the endogenous hK4-254. In view of our results of the cytoplasmic localization of hK4-254 from the GFP and V5-tagged expression systems, the cytoplasmically localized endogenous hK4 detected by the anti-hK4-N or anti-hK4-M antibody is clearly hK4-254. In addition, staining of the endogenous hK4-254 with our anti-hK4-N antibody is observed in cancer cells, but not benign glands, suggesting that its IHC expression is more cancer-specific.

In contrast to the classical hK4-254 serine protease, the hK4-205 isoform has no signal peptide and therefore will not be secreted. Recombinant hK4-205 displayed a predominantly nuclear localization on transient transfection. In addition, with the anti-hK4 C-terminal antibody on IHC and IF confocal microscopy, nuclear endogenous hK4 was detected in cultured prostate cells and prostate cancer tissue sections. The characteristics of the nuclear form reflected those of the recombinant hK4-205 in respect of cellular localization and post-translation modifications; that is, no N-glycosylation. The precise structural determinants that lead to nuclear localization of hK4-205 are not yet understood. Similarly, its precise function in the nucleus, particularly given that it also does not have a pro-region and theoretically could be constitutively active, remains to be determined.

There has been considerable controversy regarding the androgen regulation of *KLK4*/hK4 expression in LNCaP cells. Our data showed that *KLK4* mRNA levels were increased (~4-fold), but no overexpression at the protein level was observed in LNCaP cells treated by R1881, although PSA expression increased by ~10-fold at both the mRNA and protein levels. This is consistent with previous reports of *KLK4* expression in LNCaP cells treated with R1881, as assessed by northern blot analysis (Nelson *et al.* 1999) and RT-PCR analysis in the breast cancer cell line BT-474 treated by 10 nm dihydrotestosterone (Yousef *et al.* 1999). In contrast, although a similar treatment regimen was used to that in our study, *KLK4*/hK4

expression was reported by another group to be significantly increased (40-fold) in LNCaP cells treated with 10 nm R1881, as assessed by northern blot and western blot analyses (Korkmaz *et al.* 2001, Xi *et al.* 2004). The reason for this discrepancy is unclear but may reflect the different LNCaP sublines in the two laboratories.

In summary, this study has shown that *KLK4*/hK4 is expressed in prostate cancer. We further demonstrated that the endogenous full-length hK4-254 can be secreted and is predominantly cytoplasmically localized, with stronger staining in prostate cancer cells than benign glands. Thus, the full-length hK4-254, which has a secreted serine protease catalytic role, has potential as a more specific IHC marker for prostate cancer. We also provide definitive evidence that the N-terminal truncated hK4-205 is localized to the nucleus and may have a different tertiary conformation from the full-length form, as well as different post-translation modifications. As suggested previously (Xi *et al.* 2004), these data imply that the hK4-205 isoform may have a nuclear functional role that is novel for a member of this serine protease family. The precise functional roles of hK4-254 and the nuclear-localized hK4-205 isoform in prostate cancer remain to be elucidated.

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