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Kallikrein 4 (*KLK4*), A New Member of the Human Kallikrein Gene Family Is Upregulated By Estrogen and Progesterone in the Human Endometrial Cancer Cell Line, KLE.

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ABSTRACT: Endometrial cancer is the fourth most common female malignancy in women in developed countries. Estrogen, and to a lesser degree, progesterone, regulate specific target genes that are involved in endometrial tumorigenesis. A family of proteases involved in cellular proliferation, extracellular matrix degradation and thus, implicated in tumorigenesis, and regulated by estrogen and progesterone in a number of systems, are the tissue kallikreins (*KLKs*). *KLK4,* a new member of the *KLK* gene family, was found to be expressed to varying levels in a number of endometrial cancer cell lines- HEC1A, HEC1B, Ishikawa, RL95-2 and KLE- at both the mRNA and protein level. On the addition of 10 nmol/L estradiol, progesterone, or a combination of both over a 48 h period, an increase in the intracellular protein levels of K4 were observed when compared to the control (untreated) cells. We have also identified a novel *KLK4* transcript with a complete exon 4 deletion. The significance of this alternative transcript, which would give rise to a truncated protein without a serine residue (which is essential for catalytic activity), is yet to be established. These cell lines now provide a model system to study the role of *KLK4* and the molecular mechanisms of *KLK4* regulation by estrogen and progesterone, in endometrial tumorigenesis.

Endometrial cancer is the fourth most common malignancy in women in the western world (1). Estrogen and progesterone, via the regulation of specific target genes, are implicated in the pathophysiology of endometrial cancer (2). A family of such genes involved in tumorigenesis, primarily of the prostate and breast, and regulated by estrogen and progesterone, as well as androgens, in a number of systems, are the tissue kallikreins (*KLKs*) (3-5).

The *KLKs* are a highly conserved gene family of serine proteases involved in a number of physiological and pathophysiological events, such as the regulation of local blood flow, angiogenesis, cell proliferation, extracellular matrix (ECM) degradation and mitogenesis (3,5). Until recently, the human *KLK* family consisted of three genes, *KLK1-3*, but has now been extended to at least 14 genes, and for which a consistent nomenclature has recently been approved (6). One newly identified member of this family is *KLK4* (also known as prostase, KLK-L1 and PRSS17) which encodes the K4 protein (7-9). *KLK1-3* are expressed in the human endometrium (10) and implicated in various aspects of uterine function (3,10,11). In addition, *KLK1* is expressed in endometrial cancer tissue (12). Although *KLK4* is highly expressed in the prostate, we noted an expressed sequence tag (EST:4086; Accession # AA336074) from an endometrial cancer library suggesting *KLK4* is also expressed in this tumor type.

The regulation of *KLK1-3* by estrogen and progesterone has also been demonstrated in a number of systems (3-5,11,13-15). *KLK1* was shown to be regulated by estradiol in a rat pituitary tumor model (13) and the rat uterus (3,11). Levels of *KLK1* mRNA were also elevated in the human endometrium in the proliferative phase of the menstrual cycle, suggestive of estrogen regulation (14). Although primarily androgen regulated in the prostate, both *KLK2-3* are regulated by progesterone in the human breast cancer cell line, T47D (4,15). Recently, *KLK4* mRNA was also shown to be elevated by progestins in the breast cancer cell line, BT-474 (9).

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In this study we show, at the RT-PCR and protein level, that *KLK4* is expressed in a number of endometrial cancer cell lines to varying levels. Moreover, we describe a novel exon 4 deletion in the *KLK4* gene that is also expressed to varying levels in all cell lines. In addition, K4 protein levels were elevated by estrogen and progesterone treatment in the poorly differentiated KLE cell line.

Materials and Methods

Cell culture: The endometrial cancer cell lines, Ishikawa (well differentiated), HEC1A, HEC1B, RL95-2 (moderately differentiated) and KLE (poorly differentiated) and the prostate cancer cell line LNCaP, used for a control, were obtained from the American Type Culture Collection, Rockville, MD. Prostate and kidney control tissue was obtained from Drs. Frank Gardiner, (Royal Brisbane Hospital) and David Nicol, (Princess Alexandra Hospital) respectively. All cell lines were cultured in DMEM (Life Technologies, Rockville, MD) with 10% FCS, 50 U/mL Penicillin G and 50 μg/mL Streptomycin (CSL Biosciences, Melbourne, Australia) at 37C and 5% CO_2. The regulation studies were performed in triplicate in the KLE cell line using phenol-red and FCS-free media. After 24 h, fresh media was supplemented with 10 nmol/L 17β-estradiol or progesterone, (Sigma Chemical Company, St Louis, MO) and the cells were maintained for 48 h with these steroids. In addition, 10 nmol/L progesterone was added to one group of estrogen-treated cells after 24 h.

RT-PCR: Total RNA was extracted from triplicate cell preparations (10^6 cells) using TRI-Reagent (Sigma) following the manufacturers instructions. For complementary DNA (cDNA) synthesis, 2 μg of total RNA was reverse transcribed using Superscript II (Life Technologies). Primers, specific for *KLK4* (5'- GCGGCACTGGTCATGGAAAACG -3' sense, and 5'-CAAGGCCCTGCAAGTACCCG-3' antisense), and β2-microglobulin (5'-TGAATTGCTATGTG TCTGGGT-3' sense, and 5'-CCTCCATGATGCTGCTTAC AT-3' antisense) were used in a 20 μL reaction containing 100 ng/μL primers, 2.5 units of Platinum Taq (Life Technologies), 10 mmol/L deoxynucleotide triphosphates (dATP, dGTP, dCTP and dTTP), 10 X buffer containing 1.5 mmol/L Mg^{2+} (Roche, Basel, Switzerland) and 1 μL of cDNA. The PCR cycling conditions were 94C for 5 min, followed by 35 cycles at 94C for 1 min, (62C for *KLK4* and 55C for β2-microglobulin) for 1 min, and extension at 72C for 1 min. All PCR products were electrophoresed on a 2% agarose gel.

Southern analysis: Southern analysis using gene specific probes was used to verify the *KLK4* gene products. PCR products were transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Little Chalfont, UK) at room temperature for approximately 16 h. Probes for *KLK4,* exon 4 (5'-CTACCGTGCTGCAGTGCGTG-3') and exon 3 (5'-CTCCTACACCATCGGGCTGGG-3') were end-labelled with digoxigenin (DIG), added to 5 mL of DIG Easy-hyb (Roche), and incubated with the membrane overnight at 37C. Following 3 x 20 min washes and a final wash in 0.2 X SSC (20 X SSC: 3 mol/L NaCl, 30 mmol/L sodium citrate) with 0.1% SDS at 37C, the chemiluminescence substrate, CDP-Star™ (Roche), was used to record the hybridization signal.

DNA sequencing: To further confirm product specificity, a number of PCR products were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) at the DNA sequencing facility, University of Queensland, Brisbane.

Western analysis: At 80% confluency, cells were pelleted, then lysed in ice-cold lysis buffer (Tris-pH, 7.5, 10 mmol/L; 150 mmol/L NaCl; 1% triton-X 100) containing a general protease inhibitor cocktail (Roche) and centrifuged. Total cell protein (200μg) was boiled for 5 min and then electrophoresed on a 10% SDS-PAGE gel. The protein was then transferred to a nitrocellulose membrane (Protran, Schleicher and Schuell, Germany) using 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer (Sigma). The membrane was blocked in 5% skim milk overnight. Primary affinity-purified anti-peptide K4 antibody (#673305), raised in this laboratory, and secondary antibody (anti-rabbit HRP-conjugate, Roche) was diluted 1:100 and 1:1000 respectively and incubated with the membrane at room temperature for 1 h. Following secondary antibody incubation and washing, Lumi-Light Plus Western Blotting Substrate Solution (Roche) was applied directly to the membrane. Detection was determined using X-ray film and visilisation of the chemiluminescence signal. Fold changes in the level of signal were assessed using the Hewlett Packard Scan Jet IICX and ImagQuant 4.21A software (Molecular Dynamics, Amersham)

Results

KLK 4 expression in endometrial cancer cell lines: In order to confirm the RNA integrity, RT-PCR of the general "house-keeping" gene, β2-microglobulin, was used. All PCR products were of the correct size (249 bp) and thus, free of contaminating DNA (Fig.1). The expression of *KLK4* was detected in all endometrial cancer cell lines to varying degrees (Fig.1). The positive control LNCaP and kidney control (to a lesser degree) cDNA confirmed the amplification of a product of the expected size of 526 bp. A lower band, of 389 bp was also observed in all cell lines.

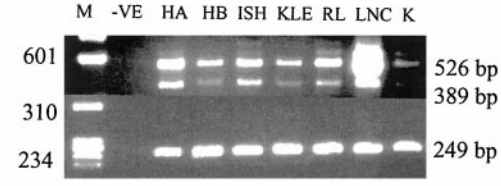

Fig. 1: RT-PCR of *KLK4* (upper panel) and β2-microglobulin (lower panel) in a number of endometrial cancer cell lines, HA (HEC1A), HB (HEC1B), ISH (Ishikawa), KLE, and RL (RL95-2). Controls include a negative (-VE, no cDNA), LNC (LNCaP) and K (Kidney). The sizes of the molecular weight marker (marker IV, Roche) and PCR products, in base pairs, are indicated at left and right respectively.

Southern and DNA Sequence Analysis: As the *KLKs* are a family of serine proteases with high sequence homology, we performed Southern blots on the RT-PCR products to confirm *KLK4* specificity. Hybridisation with an exon 3 *KLK4* probe (Fig. 2. upper panel) confirmed the expression pattern observed on the ethidium bromide stained gel. The lower 389 bp *KLK4* PCR product was sequenced and shown to be a complete exon 4 deletion (Fig. 3). Subsequent analysis with an exon 4 *KLK4* probe gave a single 526 bp band indicating that the lower band was indeed an exon 4 deletion product.

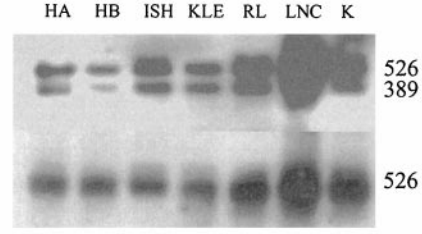

Fig. 2: Southern analysis of the ethidium bromide gel from figure 1. The upper panel was probed with an exon 3 probe resulting in two bands of 526 and 389 bp. The lower panel was probed with an exon 4 probe resulting in a single product of 526 bp.

K4 Western Analysis: Western blotting was performed to determine the relative abundance of K4 in these cell lines. Using a K4 specific antibody, we detected a protein (approx. 38-40 kDa) in all five cell lines and prostate tissue (Fig.4). The levels of K4 protein (compared to the control) were elevated by 16, 27 and 40 fold, respectively, on the addition of 10 nmol/L estradiol, progesterone, and the combination of both, in the KLE cell line over 48 h (Fig.5).

```
                    EX 1 ↓ EX 2
MATAGNPWGWFLGYLILGVA   GSLVSGSCSQIINGEDCSPHSQPWQ
MATAGNPWGWFLGYLILGVA   GSLVSGSCSQIINGEDCSPHSQPWQ

                                        ↓ EX 3
AALVMENELFCSGVLVHPQWVLSAAHCFQN   SYTIGLGLHSLEADQ
AALVMENELFCSGVLVHPQWVLSAAHCFQN   SYTIGLGLHSLEADQ

EPGSQMVEASLSVRHPEYNRPLLANDLMLIKLDESVSESDTIRSISIASQ
EPGSQMVEASLSVRHPEYNRPLLANDLMLIKLDESVSESDTIRSISIASQ

                          ↓ EX 4
CPTAGNSCLVSGWGLLAN   GRMPTVLQCVNVSVVSEEVCSKLYDP
CPTAGNSCLVSGWGLLAN   --- ----------------------------------------------

                          ↓ EX 5
LYHPSMFCAGGGQDQKDSCN   GDSGGPLICNGYLQGLVSFGKAPCG
----------------------------------------   G*

QVGVPGVYTNLCKFTEWIEKTVQAS*
```

Fig. 3: Protein sequence of the *KLK4* wild type (upper lines) and exon 4 deleted predicted product (lower lines). The five exons are marked EX1-EX5 and the exon junctions are indicated by an arrow (↓). The exon 4 deletion is indicated by a dashed line (-----). The catalytic triad, Histidine (H), Aspartic acid (D) and Serine (S), essential for catalytic activity, are marked in bold. The asterisk indicates the end of the protein sequence. For the exon 4 deleted form, this is a glycine, preceding a premature stop codon.

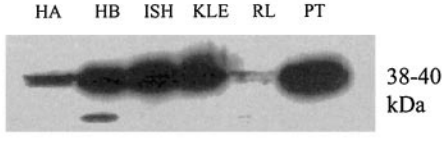

Fig. 4: Western analysis of K4 in endometrial cancer cell lines. The endometrial cancer cell lines are as noted for fig. 1, except for the last lane PT (prostate tissue). High levels of K4 intracellular protein were observed in most lines except for two moderately differentiated lines, HEC1A (HA)and RL95-2 (RL). K4 protein was observed at approximately 38-40 kDa.

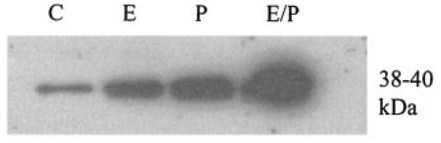

Fig. 5: K4 western analysis of 200 μg intracellular protein from the KLE cell line treated with 10 nmol/L estradiol and progesterone for 48 h. Lanes: C (control, no treatment), E (estradiol), P (progesterone) and E/P (estradiol for 24 h followed by 24 h treatment with estradiol and progesteone).

Discussion

The endometrial cancer cell lines HEC1A, HEC1B, Ishikawa, KLE and RL95-2 provide a model system to study the expression and regulation of factors that are involved in the pathological processes of endometrial cancer. Each cell line possesses a spectrum of different phenotypes and various degrees of cellular differentiation. These lines also express *KLK1-3*, and estrogen and progesterone receptors (ER and PR) to varying degrees (Myers and Clements, manuscript in preparation). Here we have identified *KLK4* mRNA in a number of endometrial cancer cell lines to varying degrees. Additionally, we have shown that K4 protein levels are elevated by estrogen and progesterone in the poorly differentiated KLE cell line.

The *KLKs* are a family of serine proteases involved in the post-translational processing of many polypeptides that are central to various physiological and pathophysiological events, including tumorigenesis (3). Recently, *KLK4* has been shown to be highly expressed in the prostate, as determined by northern blot analysis (7), and to varying levels, as shown by RT-PCR, in a number of other endocrine tissues such as the testis, adrenals, thyroid, mammary gland, and the uterus (9). Here, we extend these data and show the expression of *KLK4* in a spectrum of phenotypically different endometrial cancer cell lines, suggesting that *KLK4* is likely to be expressed in endometrial cancer tissue. Similar results were observed at the protein level, although lower K4 levels were observed in two of the moderately differentiated cell lines, HEC1A and RL95-2. The molecular mass of the K4 protein (38-40 kDa) is similar to that of the other known family members K1-3, which range from 33-43 kDa (3,5). This variation in molecular mass is due to the variable glycosylation of these enzymes (3,5). Although it is possible that our antibody may cross-react with other proteins, we feel this is highly unlikely. Two other affinity purified anti-peptide antibodies, raised against different regions of K4 also gave similar molecular sizes of approximately 38-40 kDa.

In our study, K4 protein levels were elevated by estrogen and progesterone alone, and in combination, in the KLE cell line. This was an interesting finding since the KLE cell line has been reported to harbour a defective ER (16), in that, it does not translocate to the nucleus. Our data may suggest an indirect mechanism of response, or a "non-genomic" steroid response. For example, the activation of the adenylate cyclase pathway by estrogen in rodent endometrial cells is thought to be mediated by a membrane-bound receptor (2). In addition, since these earlier studies were performed prior to the identification of two ER types, this may simply reflect the expression of perhaps ERβ and not ERα. Of interest were the combined effects of estrogen and progesterone on K4. These data suggest that estrogen and progesterone act synergistically to affect K4 expression.

To date there is little data on *KLK4* regulation and its promoter region has not been studied in any detail. LNCaP cell lines supplemented with 1 nmol/L R1881 (a synthetic androgen) for 24 hours resulted in a 50% decrease in *KLK4* steady-state levels. However, these levels rose to reach a 1.7

fold increase over a steady-state 48 hours (7). *KLK4* expression was also increased by progestins in the human breast cancer cell line BT-474 (9). Potential androgen response elements (AREs) have been identified in the 5'-flanking region of *KLK4* (8), but these have not been functionally characterised. However, given that the PR can also bind to similar response elements (4, 5) and that *KLK4* is regulated by progesterone in the breast cancer cell line BT-474 (9), it is tempting to speculate that PR may bind these response elements to regulate *KLK4* expression in endometrial cancer cell lines. Indeed, similar response elements are present in the *KLK2-3* promoters (5,15). Further work is being carried out to identify these functional regions of the 5'-flanking region of the *KLK4* gene.

The novel splice variant (exon 4 deletion) was of interest. This novel transcript, to our knowledge, has not been described before. The variant consists of 4 exons, (instead of 5) with exon 3 joining to exon 5 thus altering the reading frame. This results in a stop codon encoded by the second codon of exon 5. As this alternatively spliced transcript does not possess the essential serine residue required for catalytic activity, it is unlikely it will encode a functional enzyme. However, it will be important to determine if this transcript encodes a functional protein and is cancer-specific.

The biological function of *KLK4* is not yet known, however, K4 shares 78% homology at the amino acid level with a pig enamel matrix serine protease (EMSP1) which is involved in the degradation of the enamel protein matrix allowing the maturation of dental enamel (17). Two other new family members of the *KLK* family, *KLK6* and *KLK7* (zyme/protease M/neurosin and stratum corneum chymotryptic enzyme (SCCE) respectively) (6), have also been implicated in ECM remodelling in cancer. SCCE is a skin-specific serine protease involved in the degradation of intracellular cohesive structures in the continuous shedding of skin cells (18), and protease M is thought to be involved in the development of primary breast and ovarian tumours (19). Therefore, it is tempting to postulate that *KLK4* may also play a similar role in ECM degradation and contribute to the pathophysiological processes of endometrial cancer.

Acknowledgements

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