Down-Regulation of the Human Kallikrein Gene 5 (KLK5) in Prostate Cancer Tissues

George M. Yousef,1,2 Andreas Scorilas,3 Albert Chang,1,2 Laura Rendl,2 Maria Diamandis,2 Klaus Jung,4 and Eleftherios P. Diamandis1,2*

1Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada
2Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada
3National Center of Scientific Research “Demokritos”, IPC, Athens, Greece
4Department of Urology, University Hospital Charité, Humboldt University, Berlin, Germany

BACKGROUND. Kallikreins are a subgroup of serine proteases with diverse physiological functions. Many kallikrein genes are differentially expressed in various malignancies and prostate specific antigen (PSA; encoded by the KLK3 gene) is the best tumor marker for prostate cancer. Human glandular kallikrein (hK2; encoded by the KLK2 gene) is an emerging tumor marker for prostate cancer. KLK5 is a newly discovered human kallikrein gene which shares a high degree of homology and is located adjacent to KLK2 and KLK3 genes on chromosome 19q13.4. Like KLK2 and KLK3, the KLK5 gene is regulated by steroid hormones in the BT-474 breast cancer cell line. We have previously shown that KLK5 is differentially expressed in ovarian and breast cancer.

METHODS. We compared the expression of KLK5 in 29 pairs of histologically confirmed normal and prostate cancer tissues by quantitative RT-PCR using the LightCycler technology.

RESULTS. KLK5 expression was significantly lower in cancer tissues compared to their normal counterparts. Lowest levels of expression were found in T3 stage tumors compared with T1 and T2. Also, a significant negative correlation was found between Gleason score and KLK5 expression.

CONCLUSIONS. KLK5 should be further studied as a potential new prognostic marker in prostate cancer, whose expression is negatively correlated with cancer aggressiveness. Prostate 51: 126–132, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: kallikreins; prostate cancer; serine proteases; cancer genes; prognostic factors; predictive markers; tumor markers

INTRODUCTION

Prostate cancer is the most commonly diagnosed solid tumor in American men. Last year, approximately 179,000 new prostate cancers were diagnosed, with 37,000 deaths [1]. Early detection through serum testing for prostate specific antigen (PSA) and improved procedures for surgical intervention and radiation therapy have significantly reduced the number of fatalities [2,3]. However, non-malignant prostatic diseases, especially benign prostatic hyperplasia and prostatitis, also cause serum PSA elevation, thus complicating the diagnosis of prostatic carcinoma by PSA measurements alone [4]. Also, screening for early stage disease is limited by our inability to accurately...
predict the prognosis of a substantial proportion of localized cancers [5]. Therefore, much research has been dedicated to identify adjuvant prognostic and predictive markers which can distinguish indolent from aggressive forms of prostate cancer [6]. PSA (hK3, according to the approved new nomenclature of the human kallikrein family [7]), is a member of the human kallikrein gene family of serine proteases [8]. Besides PSA, other, structurally similar kallikrein genes may also be related to prostate cancer [9,10]. The protein encoded by the human kallikrein gene 2 (hK2) has already found applicability as an adjuvant tumor marker in subgroups of patients with moderate elevations of serum PSA [11–14]. KLK4 is highly expressed in the prostate and is under steroid hormonal regulation in prostate and breast cancer cell lines [15,16]. The human kallikrein gene 5 (KLK5), also known as kallikrein-like gene-2; (KLK-L2) [17] or human stratum corneum tryptic enzyme (HSCTE) is a recently cloned member of the human kallikrein gene family which is located adjacent to KLK4, KLK2, and PSA genes and shares a high degree of homology with these kallikreins. The gene is mainly expressed in testis, breast, brain, and epididymis [17,18]. We have recently shown that KLK5 is differentially regulated in ovarian cancer [19]. In this article we used a quantitative RT-PCR approach to compare the level of expression of KLK5 in prostate cancer tissues and their matched normal counterparts. We have also examined the relationship between KLK5 expression and standard clinicopathological variables of prostate cancer.

MATERIALS AND METHODS

Study Group

Included in this study were 29 patients who had undergone radical retropubic prostatectomy for prostatic adenocarcinoma at the Charite University Hospital, Berlin, Germany. Patients’ age ranged from 45 to 71 years with a mean of 63.5 and a median of 65 years. The patients did not receive any hormonal or other therapy before surgery.

Prostate Cancer Tissues

Fresh prostate tissue samples were obtained from the cancerous and non-cancerous parts of the same prostates. Small pieces of tissue were dissected immediately after removal of the prostate and stored in liquid nitrogen. Histological analysis was performed as previously described [20], to ensure that the tissues were either malignant or benign. The use of these tissues for research purposes was approved by the Ethics Committee of the Charite Hospital.

Total RNA Extraction and cDNA Synthesis

Tumor tissues were minced with a scalpel, on dry ice, and transferred immediately to 2 ml polypropylene tubes. They were then homogenized and total RNA was extracted using the RNaseasy® total RNA isolation system, following the manufacturer’s instructions (Qiagen, Valencia, CA). The concentration and purity of RNA were determined spectrophotometrically. Two micrograms of total RNA was reverse-transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL, Gaithersburg, MD). The final volume was 20 µl.

Quantitative Real-Time RT-PCR Analysis

Based on the published genomic sequence of KLK5 (GenBank accession # AF135028), two gene-specific primers were designed (L2-3: 5′-CAA GAC CCC CCT GGA TGT GG-3′ and 5L2: 5′-AGT TT CAG AGT CCG TCT CGG-3′). These primers spanned more than 2 exons to avoid contamination by genomic DNA.

Real-time monitoring of PCR reactions was performed using the LightCycler™ system (Roche Molecular Systems, Indianapolis, IN) and the SYBR green I dye, which binds preferentially to double stranded DNA. Fluorescence signals, which are proportional to the concentration of the PCR product, are measured at the end of each cycle and immediately displayed on a computer screen, permitting real time monitoring of the PCR reaction [21]. The reaction is characterized by the point during cycling when amplification of PCR products is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting quantity of the template, the earlier a significant increase in fluorescence is observed [22]. The threshold cycle is defined as the fractional cycle number at which fluorescence passes a fixed threshold above baseline [23].

Endogenous Control

For each sample, the amount of the target and of an endogenous control (β actin, a housekeeping gene) were determined using a calibration curve (see below). The amount of the target molecule was then divided by the amount of the endogenous reference, to obtain a normalized target value [22].

Calibration Curves

Separate calibration (standard) curves for actin and KLK5 were constructed using serial dilutions of total cDNA from healthy human prostate tissue, purchased from Clontech, Palo Alto, CA, as described by Bieche et al. [22,23]. The standard curve samples were included...
in each run. The LightCycler software automatically calculates the standard curve by plotting the starting dilution of each standard sample versus the threshold cycle, and the sample concentrations were then calculated accordingly (Fig. 1). Standards for both KLK5 and actin RNAs were defined to contain an arbitrary starting concentration, since no primary preparations exist. Hence, all calculated concentrations are relative to the concentration of the standard.

**PCR Amplification**

The PCR reaction was carried out on the LightCycler system. For each run, a master mixture was prepared on ice, containing 1 μl of cDNA, 2 μl of LC DNA Master SYBR Green I mix, 50 ng of primers, and 1.2 μl of 25 mM MgCl₂. The final volume was adjusted with H₂O to 20 μl. After the reaction mixture was loaded into a glass capillary tube, the cycling conditions were carried out as follows: initial denaturation at 94°C for 10 min, followed by 45 cycles of denaturation at 94°C for 0 sec, annealing at 60°C for 5 sec, and extension at 72°C for 16 sec. The temperature transition rate was set at 20°C per second. Fluorescent product was measured by a single acquisition mode at 86°C after each cycle.

**Melting Curve**

For distinguishing specific from non-specific products and primer dimers, a melting curve was obtained after amplification by holding the temperature...
at 70°C for 30 sec followed by a gradual increase in temperature to 98°C at a rate of 0.2°C/sec, with the signal acquisition mode set at step, as described [24]. To verify the melting curve results, representative samples of the PCR products were run on 1.5% agarose gels, purified, and cloned into the PCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

**Statistical Analysis**

Statistical analysis was performed with SAS software (SAS Institute, Cary, NC). The analyses of differences between KLK5 expression in non-cancerous and cancerous tissues were performed with the non-parametric McNemar test and Wilcoxon signed ranks test. The binomial distribution was used to compute the significance level of the McNemar test. Relationships between different variables were assessed by the Mann–Whitney U Test and Spearman correlation.

**RESULTS**

We compared the expression of the KLK5 gene in 29 pairs of prostatic tissues (normal vs. cancer, obtained from the same patient). We found a significant decrease in KLK5 expression levels in cancer tissues, compared to their normal counterparts ($P = 0.007$) (Table I). The expression levels of KLK5 were determined using arbitrary units, according to the standard curve which was constructed by using serial dilutions of cDNA obtained from normal prostatic tissue, and was maintained throughout the entire experiment. Results were further normalized by using the ratio of KLK5/actin concentration for each sample. The distribution of KLK5 mRNA concentrations in normal (non-cancerous) and cancerous tissues is shown in Table II. KLK5/actin ratios ranged from 0 to 5.68 in normal tissues, with a mean $\pm$ SD (standard deviation) of 1.70 $\pm$ 1.88 and from 0 to 3.67 in cancer tissues, with a mean $\pm$ SD of 0.65 $\pm$ 1.03. Figure 2 shows the paired values of KLK5 in non-cancerous and cancerous tissues for every patient. Nineteen out of 29 patients had higher KLK5 levels in normal tissues, while only five patients had higher KLK5 levels in cancerous tissues. In five patients, the levels of expression were comparable in normal and cancerous tissues. It should be noted, however, that in four out of five patients in the latter group, the expression was very low (less than 0.1); thus, accurate comparison between normal and malignant tissues was not feasible.

Lower expression levels of KLK5 were found in T3 stage tumors, compared with T1 and T2 stages (mean expression levels were 0.14 and 0.93, respectively) (Table III). No statistically significant association between KLK5 expression and patient age was observed (data not shown). A statistically significant association was observed between KLK5 expression and Gleason score but not with tumor grade, using the Mann–Whitney U test (Table III). However, the mean and median values are both lower in grade 3 tumors, compared with grade 1 and 2 (Table III). Using Spearman correlation, a significant negative association was found between Gleason score and KLK5 expression ($r_s = -0.557, P = 0.003$) (Fig. 3).

**DISCUSSION**

Kallikreins are a subgroup of serine proteases. Three adjacent members of the human kallikrein family, namely PSA, KLK2, and KLK4, which are grouped together in a 57 kb region of chromosome 19q13.3–q13.4, are found to be highly expressed in prostatic tissue and have been used or they are candidate prostate cancer biomarkers.

KLK5 is a new human kallikrein gene that is located 32 kb more telomeric to the KLK4 gene. We have previously shown that this gene is under steroid hormone regulation in cancer cell lines [17], and is differentially regulated in ovarian and breast tumors [19]. In this study we demonstrate that KLK5 expression is lower in prostate cancer tissues compared to their normal counterparts. Prostate cancer is known to be an androgen-dependent tumor [25] and we have previously shown that KLK5 expression is down-regulated by androgens [17]. This may provide partial explanation to our data. In addition, our results indicate that lower levels of expression correlate with higher Gleason score and late stage disease (Table III and Fig. 3).

KLK5 expression was found to be down-regulated, at the mRNA level, in prostate cancer tissues compared to normal. It should be noted, however, that the tissue expression levels might not reflect the serum levels of the protein which, at present, cannot be measured due to lack of methodology. Although levels of PSA and hK2 are elevated in the serum of

<table>
<thead>
<tr>
<th>KLK5 expression</th>
<th>No. of patients (%)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher in normal vs. cancer</td>
<td>19 (66)</td>
<td>0.007</td>
</tr>
<tr>
<td>Lower in normal vs. cancer</td>
<td>5 (17)</td>
<td></td>
</tr>
<tr>
<td>Approx. equal in both tissues</td>
<td>5 (17)</td>
<td></td>
</tr>
</tbody>
</table>

*Calculated by McNemar non-parametric test.
prostate cancer patients, their tissue concentration is lower in the prostate cancer tissues [26]. The elevation of serum concentration may be attributed to angiogenesis, destruction of the tissue architecture, and leakage of these proteins to the general circulation [27].

One of the important research areas in prostate cancer is understanding how and why prostate cancer metastasizes preferentially to the bone [6]. Although prostatic carcinoma is characterized by osteoblastic metastasis, the lesions also cause osteolysis [28,29]. The hK5 protein was shown to have proteolytic activity [18], and is structurally similar to the enamel matrix serine proteinase (EMSP) protein (68% identity at the amino acid level) [17]. EMSPs function is to degrade the enamel matrix proteins during enamel maturation [30]. Therefore, it will be interesting to study the expression levels of KLK5 in late, hormonally independent stages of prostate cancer, and to examine the possible contribution of KLK5 to bone metastasis.

The role, if any, of KLK5 in prostate cancer is still unknown. However, the down-regulation of the gene in cancer may point out to a possible inhibitory effect of the gene on cell growth. There is some evidence that PSA, a protein that is structurally similar to hK5, has a growth inhibitory effect on cancer cell lines [31], and exhibits antiangiogenic properties [32]. These proposals need experimental verification.

**CONCLUSIONS**

Our results indicate that KLK5 expression, at the mRNA level, is lower in prostate cancer tissues compared to their normal counterparts. Lowest levels of expression were found in late stage tumors, and a

| TABLE II. KLK5 Expression in Non-Cancerous and Cancerous Prostatic Tissues From 29 Patients |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                 | Mean | Standard deviation | Median | Range | P-value |
| KLK5, non-cancer                | 1.70 | 1.88              | 0.94   | 0–5.7 |         |
| KLK5, cancer                    | 0.65 | 1.03              | 0.16   | 0–3.7 | 0.006   |
| % Decrease                     | 62%  | —                 | 83%    |       |         |

*All values are expressed as ratios of KLK5 and actin concentration, as described under Materials and Methods.

*Calculated by the Wilcoxon signed ranks test.

*Calculated by assuming that value in non-cancerous tissue is 100%.

---

**TABLE III. KLK5 Expression in Cancerous Prostatic Tissues From 29 Patients Classified by Stage of the Disease, Gleason Score, and Tumor Grade**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Median</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.93</td>
<td>1.18</td>
<td>0.46</td>
<td>0.074</td>
</tr>
<tr>
<td>T3</td>
<td>0.14</td>
<td>0.25</td>
<td>0.00</td>
<td>0.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Median</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–4</td>
<td>0.98</td>
<td>1.11</td>
<td>0.47</td>
<td>0.10</td>
</tr>
<tr>
<td>5–7</td>
<td>0.47</td>
<td>0.99</td>
<td>0.10</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*Calculated by the Mann–Whitney U Test.

---

**Fig. 2.** Paired values of KLK5 mRNA expression in non-cancer (□) and cancer (■) tissues for each patient. P-value was calculated by the McNemar non-parametric test.
negative correlation between \( KLK5 \) expression and the Gleason score. \( KLK5 \) should be further studied as a potential prognostic marker for prostate cancer.

**REFERENCES**


