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# Differential Expression of the Human Kallikrein Gene 14 (*KLK14*) in Normal and Cancerous Prostatic Tissues

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**BACKGROUND.** Many members of the human kallikrein gene family are differentially expressed in cancer and a few have potential as diagnostic/prognostic markers. *KLK14* is a newly discovered human kallikrein gene that is mainly expressed in the central nervous system and endocrine tissues. Since *KLK14* was found to be regulated by steroid hormones in prostate cancer cell lines, we hypothesized that it will be differentially expressed in prostate cancer tissues compared to their normal counterparts.

**METHODS.** Matched prostate tissue samples from the cancerous and non-cancerous parts of the same prostates were obtained from 100 patients who underwent radical prostatectomy. Quantitative analysis of *KLK14* expression levels were performed by real-time RT-PCR using SYBR Green I dye on the LightCycler™ system. Associations with clinico-pathological parameters were analyzed.

**RESULTS.** *KLK14* overexpression in the cancerous compared to non-cancerous tissue was found in 74% of patients ($P < 0.001$). Mean level of expression was 154 arbitrary units (Au) in cancerous tissues and 14.2 Au in the non-cancerous tissues. The ratio of the cancerous to non-cancerous *KLK14* expression values was higher in patients with late stage (stage III) compared to stage II ($P = 0.002$), and in grade 3 compared to grade 1/2 tumors ($P = 0.001$). A statistically significant increase was also observed in patients with higher in Gleason score (>6) compared to Gleason score = 6 tumors ($P = 0.027$). No correlation was found between *KLK14* tissue expression levels and serum prostate-specific antigen.

**CONCLUSIONS.** *KLK14* expression is significantly higher in cancerous compared to non-cancerous prostatic tissue. The up-regulation of the *KLK14* gene in advanced and more aggressive tumors may indicate a possible role for the hK14 protein in tumor spread and opens the possibility of hK14 being a candidate new marker for prostate cancer diagnosis and prognosis. *Prostate 56: 287–292, 2003.* © 2003 Wiley-Liss, Inc.

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

Abbreviations: *KLK*, human kallikrein (gene); hK, human kallikrein (protein); PCR, polymerase chain reaction; PSA, prostate specific antigen; RT, reverse transcription; Au, arbitrary units.

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## INTRODUCTION

The human kallikrein family is a cluster of 15 serine protease genes on chromosome 19q13.4 [1–3]. Many kallikrein genes are differentially expressed in various malignancies [4–11]. There is a close relationship between kallikreins and the prostate gland. Both the centromeric (KLK15, KLK2, KLK3, KLK4) and the telomeric (KLK11, KLK12, KLK13, KLK14) groups of the kallikrein genes are highly expressed in the prostate [5,6,12–17]. In addition, many kallikreins were shown to be associated with prostate cancer. Prostate specific antigen (PSA; encoded by the *KLK3* gene) is the best tumor marker for prostate cancer [18], and hK2 and hK11, another two kallikreins, are emerging new biomarkers of the disease [19,20]. We have also recently shown that KLK5 is down-regulated in prostate cancer tissues compared to their normal counterparts [21].

Prostate cancer is the most common neoplasia of men in the western world. Prostate specific antigen (PSA, hK3) is the best available serum tumor marker and is currently used for prostate cancer diagnosis, prognosis, and monitoring of treatment. However, PSA lacks specificity because of its increasing levels in benign prostatic diseases [22]. In addition, other markers are needed which can distinguish between indolent and aggressive forms of the disease, and consequently, tailor-treatment decisions [23].

*KLK14* (formerly known as *KLK-L6*) is the most telomeric member of the kallikrein family [17]. This gene has a restricted tissue expression pattern and is found mainly in the central nervous system (brain, cerebellum, and spinal cord) as well as in endocrine-related tissues such as the prostate, uterus, ovary, thyroid, and testis [17,24]. Preliminary studies have shown that *KLK14* is differentially expressed, at the mRNA level, in endocrine-related malignancies [17]. In addition, in situ hybridization studies demonstrated that *KLK14* is expressed by the secretory epithelial cells of benign prostate gland, prostatic intraepithelial neoplasia, and malignant prostate cells [24]. *KLK14* was also found to be up-regulated by androgens in cancer cell lines, possibly through the androgen receptor (our unpublished data). This information led us to hypothesize that *KLK14*, like other kallkreins, may be differentially expressed in prostate cancer tissues. In this study, we investigate the expression of *KLK14* in matched normal and malignant prostate tissues by real-time quantitative RT-PCR, and examine the relationship between expression levels and other clinico-pathological parameters such as stage, grade, and Gleason score.

## MATERIALS AND METHODS

### Study Population

Matched prostate tissue samples were obtained from 100 patients (median age 63 years, range 48–75 years) who underwent radical open or laparoscopic prostatectomy for prostatic adenocarcinoma between March 1997 and April 2001 at the University Hospital Charité, Berlin, Germany. The Ethics Committee of the Charité Hospital approved the use of these tissues for research purposes. According to the TNM-system from 1997, 45 patients had stage T2 (T2a; $n = 3$, T2b; $n = 42$) and 48 patients were stage pT3 (T3a; $n = 37$, pTb (seminal vesicle invasion); $n = 11$). All operated patients were stage pN0 and M0. Fifty-six patients had a WHO grade 1 or 2 cancer and 44 had grade 3. Gleason score was $= 6$ in 46 patients and $>6$ in 47 patients, whereas it was not available in 7 patients. Positive surgical margins were found in 50 patients whereas 48 patients had negative surgical margins.

### Tissue Preparation and Primer Design

Fresh prostate tissue samples were obtained from cancerous and non-cancerous parts of prostatectomy specimens. Small pieces of tissue were gross dissected by a pathologist immediately after removal of the prostate, snap frozen and stored in liquid nitrogen until analysis. Histological analysis and confirmation for all the tissue pieces was performed by the same pathologist as described previously [25] to ensure that the tissue was either malignant or benign. The tissues were pulverized with a hammer under liquid nitrogen and RNA was extracted using the RNeasy kit (QIAGEN, Inc., Valencia, CA) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. Two μg of total RNA was reverse-transcribed into first-strand cDNA using the Superscript II preamplification system (Life Technologies, Inc., Bethesda, MD). The final volume was 20 μl. Based on the published genomic sequence of *KLK14* (Genbank accession no. AF161221), two gene-specific primers were designed (6F5: 5′ AGT GGG TCA TCA CTG CTG CT 3′ and 6R5: 5′ TCG TTT CCT CAA TCC AGC TT 3′). These primers spanned more than two exons to avoid contamination by genomic DNA.

### Quantitative RT-PCR

Quantitative RT-PCR was performed on the Roche LightCycler® system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR Green I dye, which binds preferentially to double-stranded DNA. Real time monitoring of the PCR reaction and the preparation of calibration curves were performed as described

earlier [21]. For each sample, the amounts of the target and of an endogenous control (β-actin) were determined using a calibration curve. The amount of the target molecule (*KLK14*) was then divided by the amount of the endogenous reference (β-actin) to obtain a normalized value. Separate calibration (standard) curves for β-actin and *KLK14* were constructed using serial dilutions of total cDNA from a prostate tissue pool for β-actin or by serial dilutions of a plasmid containing the *KLK14* cDNA. The plasmid for the *KLK14* was prepared according to the manufacturer's instructions with the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA). The standard curves were included in each run. The LightCycler™ software automatically calculated the standard curve by plotting the starting dilution of each standard sample versus the threshold cycle, and the sample concentrations were then calculated accordingly. Standards for both *KLK14* and β-actin cDNAs were defined to contain an arbitrary starting concentration, and serial dilutions, 10-fold each (with concentrations defined according to the dilution factor), were used to construct the standard curve.

The PCR reactions were carried out on the LightCycler™ system. For each run, a master mixture containing 1 μl of cDNA, 2 μl of Faststart DNA Master SYBR Green 1 mix, 50 ng of primers, and 2.4 μl of 25 mM $MgCl_2$ was prepared on ice. After loading the reaction mixture into glass capillary tubes, cycling conditions were carried out as shown in Table I. Each sample was analyzed twice to ensure reproducibility. To verify the melting curve results, representative PCR products were sequenced.

For β-actin, the LightCycler™ PCR reactions were carried out in a reaction mixture consisting of 15.2 μl $H_2O$, 0.8 μl (25 mM) $MgCl_2$, 0.5 μl (150 ng) of primers, 2 μl of LightCycler Fast Start DNA Master SYBR® Green I (Roche), and 1 μl of cDNA.

### Statistical Analyses

Statistical analyses were performed with SAS software (SAS Institute, Cary, NC) and SPSS 10.0 software for Windows (SPSS, Chicago, IL). Associations between clinico-pathological parameters such as stage, WHO grade, Gleason score, PSA and *KLK14* expression were analyzed by the ANOVA test, Mann–Whitney *U*-test, the Fisher's exact test, Spearman rank correlation coefficient, or the Wilcoxon signed ranks test when appropriate. Significance was defined as $P < 0.05$.

## RESULTS

The levels of *KLK14* mRNA were expressed as arbitrary units (Au), according to the standard curve, which was constructed by using serial dilutions of the *KLK14* plasmid. This standardization protocol was maintained throughout the entire experiment. Results were further normalized by using the ratio of *KLK14*/actin concentration for each sample.

### *KLKI4* Expression in Normal and Cancerous Tissues

As shown in Table II, *KLK14* expression was higher in the cancerous tissue in 74 patients (74%) and lower in 24 patients (24%, $P < 0.001$). Table III shows the distribution of *KLK14* expression in normal and cancerous tissues. Average *KLK14* (mean) expression was 11-fold higher and the median was 2.5-fold higher in the cancerous parts of the respective prostate. In 13 of the

**TABLE I. Experimental Protocol Used for Quantitative PCR Amplification of the *KLKI4* Gene**

| Segment number | Temperature target (°C) | Hold time (sec) | Slope (°C/sec) | Application mode |
|---|---|---|---|---|
| Program: Denaturation | | | Cycles: 1 | |
| 1 | 95 | 600 | 20 | None |
| Program: PCR | | | Cycles: 35 | |
| 1 | 95 | 0 | 20 | None |
| 2 | 62 | 5 | 20 | None |
| 3 | 72 | 45 | 20 | None |
| 4 | 85 | 5 | 20 | Single |
| Program: Melting | | | Cycles: 1 | |
| 1 | 95 | 0 | 20 | None |
| 2 | 72 | 30 | 20 | None |
| 3 | 97 | 0 | 0.2 | Step |
| Program: Cooling | | | Cycles: 1 | |
| 1 | 40 | 30 | 1 | None |

**TABLE II. *KLKI4* Expression in Pairs of Non-Cancerous and Cancerous Prostatic Tissues**

| *KLK14* expression (Au) | Number of patients (%) | *P* value* |
|---|---|---|
| Higher in normal vs. cancer | 74 (74.0) | <0.001 |
| Lower in normal vs. cancer | 24 (24.0) | |
| Equal | 2 (2.0) | |

*Calculated by Wilcoxon signed ranks test.

24 patients where *KLK14* expression was lower in the cancerous part, the expression exceeded 50% of the expression in the normal part of the gland, suggesting only a modest down-regulation.

### KLKI4 Expression in Relation to Other Variables

*KLK14* expression was observed in early stage and less aggressive tumors. Higher expression levels of *KLK14* were found in stage III tumors (mean expression level 256 and median 8.6 Au), compared to stage II (mean 38 Au and median 4.2 Au) (Table IV). The mean and median levels of *KLK14* expression were also considerably higher in grade 3 tumors (307 and 12.3 Au, respectively) compared grade 1 or 2 tumors (31 and 4.4 Au, respectively). Similar results were seen in patients with positive surgical margins compared with those with negative margins, and in patients with Gleason score >6 compared to those with a score of = 6 (Table IV). These results, however, were not statistically significant. When comparing the ratio of *KLK14* expression in cancer/non-cancerous tissues (Table V), a significant increase was found in grade 3 tumors compared with grade 1 and 2 (mean of 22 and 3.1 Au, respectively, and $P = 0.001$). Also, stage III patients showed higher expression levels compared with stage II (mean of 19 and 6.1, respectively, and $P = 0.002$), and in patients with positive surgical margins ($P = 0.001$). Patients with high Gleason scores (>6) were also expressing higher levels of *KLK14* (mean = 20 and median = 3.0 compared to a mean of 3.5 and median 1.4, respectively, $P = 0.027$). No statistically significant association was observed between *KLK14* expression and patient age (data not shown).

No correlation was found between serum PSA levels and *KLK14* mRNA expression (data not shown).

## DISCUSSION

The usefulness of hK3 (PSA) as tumor marker for prostate cancer is well established [18,22]. hK2, another member of the newly expanded kallikrein family, may add useful information in reducing unnecessary biopsies [26]. However, hK3 levels below 10 μg/L cannot predict accurately the presence of prostate cancer or the rate of postoperative PSA failure. There is a need for new and better prostate cancer markers, especially for Gleason 4/5 grade tumors [23].

The differential expression of *KLK14* in prostate cancer is not unprecedented. In addition to PSA (hK3) and hK2 which are differentially-regulated in prostate cancer, we have recently shown that another new kallikrein gene, *KLK15*, is up-regulated in prostate tumors compared to normal tissue of the same gland [12], while *KLK5* is down-regulated [21]. It will be interesting to examine the pattern of expression of all these kallikreins in prostate cancer and determine if their combination constitutes a novel prognostic/predictive panel. Little is known about the mechanism of involvement of kallikreins in cancer. Hormonal regulation might be a factor (see below). In addition, there is now growing evidence suggesting a ''cross talk'' between kallikreins and participation in common pathways that affect normal physiological or pathological processes [27].

Our finding of *KLK14* gene overexpression in prostate cancer is consistent with our earlier observations indicating that *KLK14* is up-regulated by androgens, likely through androgen receptor pathways. Prostate

**TABLE III. Descriptive Statistics of *KLKI4* Expression in Cancer and Non-Cancer Tissues**

| | | | Centiles | | | | |
|---|---|---|---|---|---|---|---|
| *KLK14* expression (Au) | Mean ± SE[a] | Range | 10 | 25 | 50 (Median) | 75 | 90 |
| Cancer tissues (N = 100) | 153 ± 91 | 0.01–8800 | 0.50 | 1.90 | 7.7 | 27 | 119 |
| Non-cancer tissues (N = 100) | 14.2 ± 5.1 | 0.00–491 | 0.28 | 0.81 | 3.1 | 9.6 | 29 |
| Cancer/non-cancer ratio (N = 100) | 12.7 ± 4.8 | 0.11–42 | 0.52 | 1.00 | 2.15 | 4.9 | 15.4 |

[a]Standard error.

**TABLE IV. *KLK14* Expression in 100 Patients Classified by Stage, Gleason Score, and Tumor Grade**

| | Total | Mean[a] | Standard error[a] | Median[a] | *P* value[b] |
|---|---|---|---|---|---|
| Grade | | | | | |
| G1/2 | 56 | 31 | 15 | 4.5 | 0.086 |
| G3 | 44 | 308 | 207 | 12.4 | |
| Stage | | | | | |
| II | 47 | 38 | 18 | 4.3 | |
| III | 53 | 256 | 172 | 8.7 | 0.17 |
| Surgical margin | | | | | |
| Negative | 48 | 94 | 58 | 3.9 | |
| Positive | 50 | 215 | 176 | 8.9 | 0.13 |
| Unknown | 2 | | | | |
| Gleason score | | | | | |
| ≤6 | 46 | 30 | 18 | 4.3 | |
| >6 | 47 | 293 | 194 | 10.5 | 0.098 |
| Unknown | 7 | | | | |

[a]mRNA levels (Au).
[b]Calculated by the Mann–Whitney *U*-test.

cancer is a known hormone-related malignancy [28], and steroid hormone receptor signaling plays a vital role in all stages of prostate carcinogenesis [29].

It is now widely accepted that no single biomarker will provide all the necessary information for diagnosis, prognosis, and development of treatment strategies in cancer patients. Instead, research is now focussing on devising panels of cancer biomarkers. Artificial network and other combinatorial approaches seem to be promising in this regard. The overexpression of *KLK14* in prostate cancer tissue suggests that it may be a good candidate biomarker, both at the mRNA or serum protein levels.

In conclusion, our results indicate that the *KLK14* gene is up-regulated in prostate cancer tissues, and higher expression levels correlate with advanced and more aggressive tumors. This may indicate a possible role for the hK14 protein as a future diagnostic and prognostic biomarker. Furthermore, the understanding of the biological function of this protein in prostatic tissue may help in delineating its role in prostate physiology and pathobiology.

**TABLE V. *KLK14* Expression (Ration Between Cancer and Non-Cancer Tissues) in 100 Patients Classified by Stage, Gleason Score, and Tumor Grade**

| | Total | Mean[a] | Standard error[a] | Median[a] | *P* value[b] |
|---|---|---|---|---|---|
| Grade | | | | | |
| G1/2 | 56 | 3.1 | 0.7 | 1.4 | 0.001 |
| G3 | 44 | 22 | 10 | 3.7 | |
| Stage | | | | | |
| II | 47 | 6.1 | 3.2 | 1.2 | 0.002 |
| III | 53 | 19 | 8.6 | 3.4 | |
| Surgical margin | | | | | |
| Negative | 48 | 6.2 | 3.1 | 1.4 | |
| Positive | 50 | 19 | 9.1 | 3.6 | 0.001 |
| Unknown | 2 | | | | |
| Gleason score | | | | | |
| ≤6 | 46 | 3.5 | 0.8 | 1.4 | 0.027 |
| >6 | 47 | 20 | 9.6 | 3.0 | |
| Unknown | 7 | | | | |

[a]mRNA levels (Au).
[b]Calculated by the Mann–Whitney *U*-test.

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