

## QUANTITATIVE ANALYSIS OF KALLIKREIN 15 GENE EXPRESSION IN PROSTATE TISSUE

CARSTEN STEPHAN,\* GEORGE M. YOUSEF, ANDREAS SCORILAS, KLAUS JUNG,†  
MONIKA JUNG, GLEN KRISTIANSEN, STEFFEN HAUPTMANN, BHUPINDER S. BHARAJ,  
TERUKAZU NAKAMURA, STEFAN A. LOENING AND ELEFTHERIOS P. DIAMANDIS

*From the Departments of Urology and Pathology, University Hospital Charité, Humboldt University, Berlin, Germany, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada, and National Center for Scientific Research "Demokritos," Athens, Greece*

### ABSTRACT

**Purpose:** The newly discovered human kallikrein 15 gene KLK15 has been shown in preliminary analysis to be associated with more aggressive types of prostate cancer. We quantitatively measured and compared gene expression of KLK15 in malignant and benign prostate tissues.

**Materials and Methods:** Matched prostate tissue samples from the cancerous and noncancerous parts of the same prostates were obtained from 90 patients who underwent radical prostatectomy. Quantitative reverse transcriptase-polymerase chain reaction using SYBR Green I and the LightCycler system (Roche Applied Science, Mannheim, Germany) was performed. Associations of KLK15 expression with clinicopathological parameters were analyzed.

**Results:** KLK15 over expression in cancerous versus noncancerous tissue was found in 76 of the 90 patient samples (84.4%,  $p < 0.001$ ). The ratio of cancerous-to-noncancerous KLK15 expression tended to be higher in patients with stage pT3/4 versus pT2 tumors ( $p = 0.1$ ). KLK15 expression tended to be higher in grade 3 than in grade 2 tumors and in Gleason score 7 or greater than in Gleason score less than 7 tumors ( $p = 0.18$  and  $0.23$ , respectively). A 1.7 cutoff at the 40th percentile provided a significant difference in stages pT2 and pT3/4 tumors ( $p = 0.029$ ).

**Conclusions:** On quantitative real-time polymerase chain reaction KLK15 expression was significantly higher in cancerous than in noncancerous tissue. Up-regulation of the KLK15 gene in advanced and more aggressive tumors may indicate a possible role for KLK15 protein as future serum marker for prostate cancer and for distinguishing tumor aggressiveness.

**KEY WORDS:** prostate; prostatic neoplasms; gene expression; tumor markers, biological; kallikreins

Prostate cancer is the most common neoplasm in men in Western countries. Prostate specific antigen (PSA) is the best available serum tumor marker. However, PSA lacks specificity because of increasing levels in benign prostate diseases.<sup>1</sup> The determination of free PSA and its ratio to total PSA is now clinically established and it is used to reduce the number of unnecessary prostate biopsies.<sup>1,2</sup> Furthermore, it has been shown that human glandular kallikrein 2 (hK2) may add significant information to distinguish prostate cancer and benign prostate hyperplasia in the PSA range of 2 to 10 ng/mL.<sup>3,4</sup> PSA and hK2 are serine proteases and their related genes KLK3 and KLK2 belong to the same gene family, namely the human tissue kallikrein gene family. In the last 3 to 4 years 12 new members of this family have been identified.<sup>5</sup> All 15 kallikrein genes share significant homologies and are clustered within a 300 kb region on human chromosome 19q13.4. These kallikreins KLK1 to KLK15, encoding for hK1 to hK15, respectively, were named with a new nomenclature.<sup>6</sup>

A recently described new kallikrein gene is KLK15, which is the centromeric neighbor of the PSA gene, whereas KLK2 is the telomeric neighbor.<sup>7</sup> Preliminary analysis showed that this gene is up-regulated in prostate tumor tissue compared with normal tissue of the same prostate gland.<sup>7</sup> In contrast,

PSA is down-regulated in cancerous tissue compared with normal tissue at the mRNA<sup>8</sup> and protein<sup>9</sup> levels. In cancerous tissue hK2 also shows down-regulation.<sup>9</sup> The strong correlation of PSA with the hK2 concentration in malignant and benign tissue suggests that these kallikreins may be regulated by similar mechanisms.<sup>9</sup> Interestingly Takayama et al showed that hK15, which was named prostin, can better activate conversion of the proform of PSA, pro-PSA, to active PSA than hK2.<sup>10</sup> This finding implicates a possible important physiological role of KLK15 for regulating PSA activity.

We investigated KLK15 expression in malignant and benign matched prostate tissue by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Based on our preliminary results from qualitative expression analysis of KLK15 in matched prostate tissue<sup>7</sup> and from quantitative analysis of the prostate gene PART-1<sup>11</sup> we measured this new kallikrein gene in a large cohort to examine the possible relationships with stage, grade and other parameters using new real-time PCR technology, that is a LightCycler.

### MATERIALS AND METHODS

**Study population.** Matched prostate tissue samples were obtained from 90 patients with a median age of 63 years (range 50 to 75) who underwent radical open or laparoscopic prostatectomy for prostatic adenocarcinoma between March 1997 and April 2001 at University Hospital Charité, Berlin, Germany. For at least 2 to 4 weeks before surgery hormonal therapy was administered in 10 of the 90 patients who were also analyzed separately, whereas the remaining 80 did not receive any hormonal therapy. The ethics committee at

Accepted for publication May 3, 2002.

Supported by MSD Pharmaceuticals, Grant 400770 from Funds of the German Chemical Industry and Sonnenfeld-Stiftung.

\* Financial interest and/or other relationship with MSD Pharmaceuticals.

† Financial interest and/or other relationship with Foundations Funds of Chem. Jud.

Charité Hospital approved the use of these tissues for research purposes. A total of 44 cases were stage pT2, 45 were stage pT3 and 1 was stage pT4 (positive margin at bladder neck). WHO grade was 2 and 3 in 53 and 37 patients, respectively. Gleason score was less than 7 in 41 and 7 or greater in 43 patients, whereas it was not available in 6. Positive surgical margins were noted in 45 patients, whereas 45 had negative surgical margins.

**Tissue preparation and primer design.** Fresh prostate tissue samples were obtained from the cancerous and noncancerous parts of prostatectomy specimens. Small pieces of tissue were grossly dissected by an experienced pathologist (G. K.) immediately after prostate removal, snap frozen and stored in liquid nitrogen until analysis, as described previously.<sup>12</sup> Because only 1 pathologist was involved in tissue selection, we could only include a subset of all patients who underwent surgery in this period. Histological analysis of paraffin embedded tissue was performed by a single pathologist to verify the diagnoses. Only tumor samples that were completely surrounded by malignant tissue according to this analysis were used in this study. We also did not include any samples in which benign prostate glands were more than 10% of the total mass. In this way contamination of the tumor samples with benign prostate glands was minimized. Most tumors were located dorsolateral in the prostate peripheral zone. Tissue characterized as normal was usually obtained from the inner zone of the contralateral lobe. Histologically many of these samples showed mild glandular hyperplasia. Study exclusion criteria were prominent inflammatory infiltrates, lack of epithelium due to stromal hyperplasia and prostatic intraepithelial neoplasia.

Tissues were pulverized with a hammer under liquid nitrogen. RNA was extracted using an RNeasy kit (Qiagen, Valencia, California) according to manufacturer instructions. RNA concentration was determined by spectrophotometry. Total RNA (2  $\mu$ g.) were reverse transcribed into first strand cDNA using the Superscript II pre-amplification system (Life Technologies, Rockville, Maryland). In addition to previously described primers for KLK15,<sup>7</sup> new primers were designed that amplify a specific product of 452 bp for KLK15 (GenBank accession No. AF242195). The primer sequences were forward 5'-TGTGGCTTCTCCTCACTCC-3' and reverse 5'-AGGCTCGTTGTGGGACAC-3'. Primers for  $\beta$ -actin were used as described previously.<sup>11</sup>

**Quantitative RT-PCR.** Quantitative RT-PCR was performed on the LightCycler instrument using SYBR Green I dye. The real-time PCR reaction and preparation of calibration curves as well as estimation of intrapatient variation were performed as described earlier.<sup>13</sup> For each sample the amounts of the target and the housekeeping gene ( $\beta$ -actin) were determined. The ratio of KLK15-to- $\beta$ -actin was calculated as the normalized value. The PCR reaction mixture for KLK15 consisted of 12.6  $\mu$ l. water, 2.4  $\mu$ l. (4 mM.)  $MgCl_2$ , 0.5  $\mu$ l. (200 ng.) primers, 2  $\mu$ l. LightCycler Fast Start DNA Master SYBR Green I and 2  $\mu$ l. cDNA. Cycling conditions were initial denaturation at 95C for 10 minutes, followed by 32 cycles of denaturation at 95C for 1 second, annealing at 64C for 4 seconds and extension at 72C for 25 seconds. The temperature transition rate was set at 20C per second. The fluorescent signal was acquired at 92C for 4 seconds after each cycle.

For  $\beta$ -actin the PCR reaction mixture consisted of 15.2  $\mu$ l. water, 0.8  $\mu$ l. (2 mM.)  $MgCl_2$ , 0.5  $\mu$ l. (150 ng.) primers, 2  $\mu$ l. of LightCycler Fast Start DNA Master SYBR Green I and 1  $\mu$ l. cDNA. Cycling conditions were initial denaturation at 95C for 10 minutes, followed by 35 cycles of denaturation at 95C for 0 seconds, annealing at 62C for 5 seconds and extension at 72C for 40 seconds. Fluorescence was acquired at 85C for 5 seconds after each cycle. To demonstrate the reliability of the whole measurement procedure (RNA isolation, cDNA synthesis and RT-PCR) and intrapatient variation 3 to 5 separate meas-

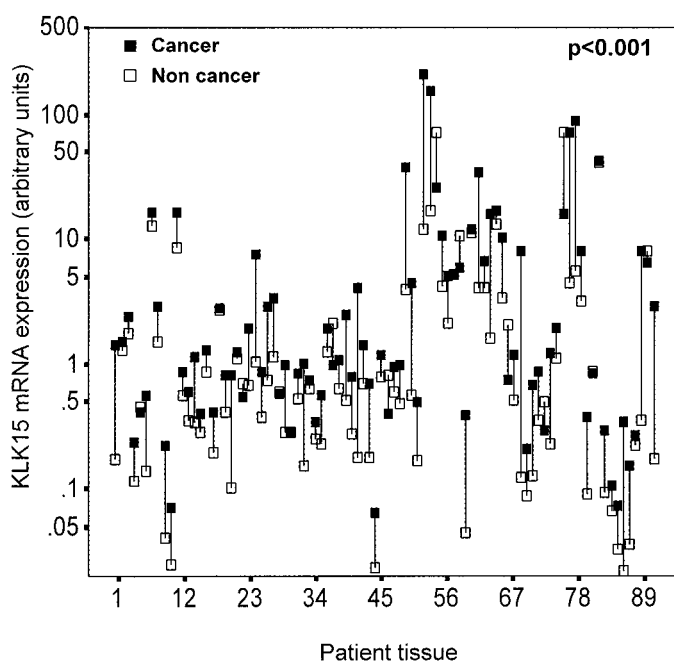
urements were performed in samples of 7 patients. The coefficient of variation was between 10% and 16%.

**Statistical assessment.** Statistical analyses were performed with SAS software (SAS Institute, Cary, North Carolina) and SPSS 10.0 software for Windows (SPSS, Chicago, Illinois). Associations of clinicopathological parameters, such as stage, WHO grade, Gleason score, PSA, percent free PSA and KLK15 expression, were analyzed by ANOVA, the Mann-Whitney U test, the Fisher exact test, the Spearman rank correlation coefficient or the Wilcoxon signed ranks test as appropriate. An optimal cutoff point was adjusted to find possible differences in clinicopathological parameters. Significance was considered at  $p < 0.05$ .

## RESULTS

Comparing the 10 patients (KLK15 over expression in 9) who received antiandrogen therapy before radical prostatectomy with the remaining 80 (KLK15 over expression in 67) without any therapy before surgery revealed a significant difference only in median preoperative PSA concentration (1.25 versus 8.0 ng./ml.,  $p = 0.003$ ). All other parameters, including  $\beta$ -actin expression ( $p = 0.87$ ), KLK15 ( $p = 0.69$ ) and the ratio of KLK15-to- $\beta$ -actin ( $p = 0.36$ ), showed no differences in the 2 groups (all  $p$  between 0.15 and 0.8). Therefore, we considered all patients as a single group of 90. Since each KLK15 value was divided by the endogenous control value ( $\beta$ -actin) to determine the normalized value, this ratio was used as an aggregate of KLK15 expression. All KLK15 value determinations were performed in duplicate.

The figure shows that KLK15 expression was higher in cancerous tissue in 76 patients (84.4%) and lower in 14 (15.6%,  $p < 0.001$ ). An expression value of greater than 1 indicates higher expression in the cancerous than in the noncancerous part of the prostate gland. Mean KLK15 expression was 4.7-fold (median 2.28-fold) higher in the cancerous parts of the respective prostates. In the 76 patients with higher KLK15 expression in the cancerous part over expression was up to 2-fold in 26, between 2 and 5-fold in 29, between 5 and 15-fold in 13 and more than 15-fold in 8. Absolute KLK15 values without correcting for  $\beta$ -actin simi-



KLK15 mRNA expression in 90 patients. Black boxes represent level in cancerous tissue. White box represent level in nonmalignant tissue in same patients. Wilcoxon signed rank test  $p$  value.

larly showed higher expression in the cancerous part in 75 patients and lower expression in 15 ( $p < 0.001$ ).

In the 90 patients median age was 63 years, median preoperative PSA was 7.6 ng./ml., median percent free PSA was 7.8% and median prostate volume was 29 ml. Table 1 lists KLK15 expression values in relation to stage, grade, Gleason score and surgical margin status. Patients with stages pT3 and pT4 disease showed a trend toward higher values than those with stage pT2 disease ( $p = 0.1$ ). Similarly KLK15 expression tended to be higher in grade 3 versus grade 2 tumors ( $p = 0.18$ ) and in Gleason score 7 or greater versus less than 7 tumors ( $p = 0.23$ ). Patients with positive surgical margins showed higher KLK15 values than those with negative surgical margins ( $p = 0.045$ ).

KLK15 tissue expression did not correlate with serum PSA (Spearman rank correlation coefficient  $r_s = [\text{minus}]0.084$ ,  $p = 0.44$ ) or with percent free PSA ( $r_s = -0.063$ ,  $p = 0.59$ ). There was also no correlation of KLK15 tissue expression with age ( $r_s = -0.065$ ,  $p = 0.54$ ) or prostate volume ( $r_s = -0.054$ ,  $p = 0.62$ ).

By searching for an appropriate cutoff we found a significant difference in stage pT2 and pT3/4 tumors at a cutoff equal to the 40th percentile (KLK15 expression 1.7,  $p = 0.029$ ). At this point only 50% of stage pT2 cases were above this cutoff, whereas 74% of stage pT3/4 cases had values above this point. Grade and Gleason score did not achieve significance ( $p = 0.27$  and  $0.26$ , respectively, table 2).

This study showed over expression of the new gene KLK15 in cancerous prostate tissue compared with normal tissue of the same gland in 84% of all cases independent of preoperative treatment. This over expression was not related to known serum biomarkers and seemed to be weakly associated with higher stage, grade and more aggressive disease.

#### DISCUSSION

The usefulness of PSA as tumor marker for prostate cancer is well established. Another member of the newly expanded kallikrein family, hK2, may add useful information for reducing unnecessary biopsies.<sup>2</sup> Nevertheless, these serum markers cannot predict 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.<sup>14</sup>

The principle of developing new biomarkers was recently speculated to be a 5-phase process.<sup>15</sup> This principle should be considered in further searches for new biomarkers. Recently developed immunoassays for new kallikrein proteins have been applied for diagnosing other cancers (ovarian) and other diseases (Alzheimer's).<sup>5</sup> A recently described new kallikrein gene is KLK15.<sup>7</sup> It was postulated that this gene is up-regulated in prostate tumors compared with normal tissues of the same prostate gland.<sup>7</sup> However, these data were ob-

TABLE 2. Relationship of KLK15 expression status and pathohistological parameters

Variable	No. Pts.	No. KLK15 Neg. (%) <sup>*</sup>	No. KLK15 Pos. (%) <sup>*</sup>	p Value (Fisher's exact test)
Stage:				0.029
pT2	44	22 (50)	22 (50)	
pT3/4	46	12 (26.1)	34 (73.9)	
Gleason score:				0.26
Less than 7	41	19 (46.3)	22 (53.7)	
7 or Greater	43	14 (32.6)	29 (67.4)	
Unknown	6			
Grade:				0.27
2	53	23 (43.4)	30 (56.6)	
3	37	11 (29.7)	26 (70.3)	

Cutoff point 1.7 arbitrary units, equal to 40th percentile.

Levels less than 1.7 and 1.7 or greater considered KLK15 negative and positive, respectively.

tained in a small number of patients and not from quantitative PCR. Our study extends the data on KLK15 expression in a large cohort with quantitative measurements. We were able to confirm over expression of this gene in cancerous parts of the prostate. In the study of Yousef et al there was KLK15 over expression in 13 of 29 patients (45%).<sup>7</sup> This study showed over expression in 76 of 90 patients (84%) and more than 2-fold over expression in 50 (56%). This over expression is also evident in patients with antiandrogen deprivation before radical surgery. Although KLK15 was found to be up-regulated by steroid hormones in the LNCaP prostate cancer cell line,<sup>7</sup> we noted no difference in absolute tissue expression levels in patients with and without preoperative antiandrogen treatment. However, the number of patients who underwent androgen deprivation was small (10) and a conclusion about protein expression levels is not currently possible due to the lack of corresponding antibodies.

Another aspect relates to the mRNA level of the respective genes in the human tissue kallikrein family (PSA, KLK2 and KLK15) and protein expression of their proteins (PSA, hK2 and hK15). In contrast to KLK15, it is known that PSA is down-regulated in cancerous tissue compared with normal tissue at the mRNA<sup>8,16</sup> and protein<sup>9,16</sup> levels. At the protein level hK2 also shows this down-regulation in cancerous tissue.<sup>9</sup> A recent study could not verify these results, showing higher KLK2 values at the mRNA level and higher hK2 levels at the protein level in malignant prostate tissue compared with benign tissue.<sup>16</sup> Earlier studies using immunohistochemical methods also showed that hK2 was over expressed in cancerous tissue.<sup>17</sup> Over expression of KLK15 in cancerous tissue suggests that hK15 may be a good candidate serum marker for prostate cancer.<sup>15</sup>

The functional relationships of some members of the kallikrein family have been investigated. It was shown that hK2 can activate pro-PSA to PSA.<sup>18,19</sup> Furthermore, it was more recently demonstrated that hK2 with its trypsin-like specificity was unable to hydrolyze the pro-PSA substrate, raising the possibility that an additional processing protease may be required to process PSA completely to an enzymatically active form.<sup>20</sup> Prostasin, which is identical to hK15, can better catalyze the conversion of pro-PSA to active PSA than hK2.<sup>10</sup> This finding implicates a possible physiological role of hK15 for regulating PSA activity.

#### CONCLUSIONS

Up-regulation of the KLK15 gene in prostate cancer, and in advanced and more aggressive prostatic tumors may indicate a possible role for hK15 protein as a future diagnostic and prognostic biomarker. Furthermore, understanding the biological function of this protein in prostate tissue may help delineate its role in prostate physiology and pathobiology.

Silke Klotzek and Sabine Becker provided technical assistance.

TABLE 1. KLK15 expression in cancerous tissues from 90 patients classified by disease stage, Gleason score and tumor grade parameters

	No. Pts.	Median KLK15/ β-Actin mRNA	p Value (Mann-Whitney U test)
Stage:			0.1
pT2	44	1.72	
pT3/4	46	2.44	
Gleason score:			0.23
Less than 7	41	2.07	
7 or Greater	43	2.38	
Unknown	6		
Grade:			0.18
2	53	2.03	
3	37	2.78	
Surgical margins:			0.045
Neg.	45	1.99	
Pos.	45	2.52	



## REFERENCES

- Polascik, T. J., Oesterling, J. E. and Partin, A. W.: Prostate specific antigen: a decade of discovery—what we have learned and where we are going. *J Urol*, **162**: 293, 1999
- Stephan, C., Jung, K., Lein, M., Sinha, P., Schnorr, D. and Loening, S. A.: Molecular forms of prostate-specific antigen and human kallikrein 2 as promising tools for early diagnosis of prostate cancer. *Cancer Epidemiol Biomarkers Prev*, **9**: 1133, 2000
- Nam, R. K., Diamandis, E. P., Toi, A., Trachtenberg, J., Magklara, A., Scorilas, A., Papnastasiou, P. A. et al: Serum human glandular kallikrein-2 protease levels predict the presence of prostate cancer among men with elevated prostate-specific antigen. *J Clin Oncol*, **18**: 1036, 2000
- Partin, A. W., Catalona, W. J., Finlay, J. A., Darte, C., Tindall, D. J., Young, C. Y. et al: Use of human glandular kallikrein 2 for the detection of prostate cancer: preliminary analysis. *Urology*, **54**: 839, 1999
- Yousef, G. M. and Diamandis, E. P.: The new human tissue kallikrein gene family: structure, function, and association to disease. *Endocr Rev*, **22**: 184, 2001
- Diamandis, E. P., Yousef, G. M., Clements, J., Ashworth, L. K., Yoshida, S., Egelrud, T. et al: New nomenclature for the human tissue kallikrein gene family. *Clin Chem*, **46**: 1855, 2000
- Yousef, G. M., Scorilas, A., Jung, K., Ashworth, L. K. and Diamandis, E. P.: Molecular cloning of the human kallikrein 15 gene (KLK15). Up-regulation in prostate cancer. *J Biol Chem*, **276**: 53, 2001
- Henttu, P., Lukkariinen, O. and Vihko, P.: Expression of the gene coding for human prostate-specific antigen and related hGK-1 in benign and malignant tumors of the human prostate. *Int J Cancer*, **45**: 654, 1990
- Magklara, A., Scorilas, A., Stephan, C., Kristiansen, G. O., Hauptmann, S., Jung, K. et al: Decreased concentrations of prostate-specific antigen and human glandular kallikrein 2 in malignant versus nonmalignant prostatic tissue. *Urology*, **56**: 527, 2000
- Takayama, T. K., Carter, C. A. and Deng, T.: Activation of prostate-specific antigen precursor (pro-PSA) by prostin, a novel human prostatic serine protease identified by degenerate PCR. *Biochemistry*, **40**: 1679, 2001
- Sidiropoulus, M., Chang, A., Jung, K. and Diamandis, E. P.: Expression and regulation of prostate androgen regulated transcript-1 (PART-1) and identification of differential expression in prostate cancer. *Br J Cancer*, **85**: 393, 2001
- Meyer, A., Jung, K., Lein, M., Rudolph, B., Schnorr, D. and Loening, S. A.: Factors influencing the ratio of free to total prostate-specific antigen in serum. *Int J Cancer*, **74**: 630, 1997
- Yousef, G. M., Kyriakopoulou, L. G., Scorilas, A., Fracchioli, S., Ghiringhello, B., Zarghooni, M. et al: Quantitative expression of the human kallikrein gene 9 (KLK9) in ovarian cancer: a new independent and favorable prognostic marker. *Cancer Res*, **61**: 7811, 2001
- Stamey, T. A.: Preoperative serum prostate-specific antigen (PSA) below 10 microg/l predicts neither the presence of prostate cancer nor the rate of postoperative PSA failure. *Clin Chem*, **47**: 631, 2001
- Sullivan Pepe, M., Etzioni, R., Feng, Z., Potter, J. D., Thompson, M. L., Thornquist, M. et al: Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst*, **93**: 1054, 2001
- Herrala, A. M., Porvari, K. S., Kyllonen, A. P. and Vihko, P. T.: Comparison of human prostate specific glandular kallikrein 2 and prostate specific antigen gene expression in prostate with gene amplification and overexpression of prostate specific glandular kallikrein 2 in tumor tissue. *Cancer*, **92**: 2975, 2001
- Darson, M. F., Pacelli, A., Roche, P., Rittenhouse, H. G., Wolfert, R. L., Saeid, M. S. et al: Human glandular kallikrein 2 expression in prostate adenocarcinoma and lymph node metastases. *Urology*, **53**: 939, 1999
- Takayama, T. K., Fujikawa, K. and Davie, E. W.: Characterization of the precursor of prostate-specific antigen. Activation by trypsin and by human glandular kallikrein *J Biol Chem*, **272**: 21582, 1997
- Kumar, A., Mikolajczyk, S. D., Goel, A. S., Millar, L. S. and Saedi, M. S.: Expression of pro form of prostate-specific antigen by mammalian cells and its conversion to mature, active form by human kallikrein 2. *Cancer Res*, **57**: 3111, 1997
- Denmeade, S. R., Lovgren, J., Khan, S. R., Lilja, H. and Isaacs, J. T.: Activation of latent protease function of pro-hK2, but not pro-PSA, involves autoprocessing. *Prostate*, **48**: 122, 2001