The Prostate 51:126 – 132 (2002)

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. Diamandis[1,2]*

[1]*Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada*
[2]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada*
[3]*National Center of Scientific Research "Demokritos", IPC, Athens, Greece*
[4]*Department 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.*

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

Abbreviations used: KLK, kallikrein; PCR, polymerase chain reaction; PSA, prostate specific antigen; hK2, human glandular kallikrein protein; RT, reverse transcription; HSCTE, human stratum corneum tryptic enzyme; EMSP, enamel matrix serine proteinase; hK5, human kallikrein 5 protein; KLK5, human kallikrein gene 5.

*Correspondence to: Dr. Eleftherios P. Diamandis, Mount Sinai Hospital, Department of Pathology and Laboratory Medicine, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada.
E-mail: ediamandis@mtsinai.on.ca
Received 27 July 2001; Accepted 4 December 2001
DOI 10.1002/pros.10067

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 epidermis [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 RNeasy® 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 TTT 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 $MgCl_2$. The final volume was adjusted with H_2O 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

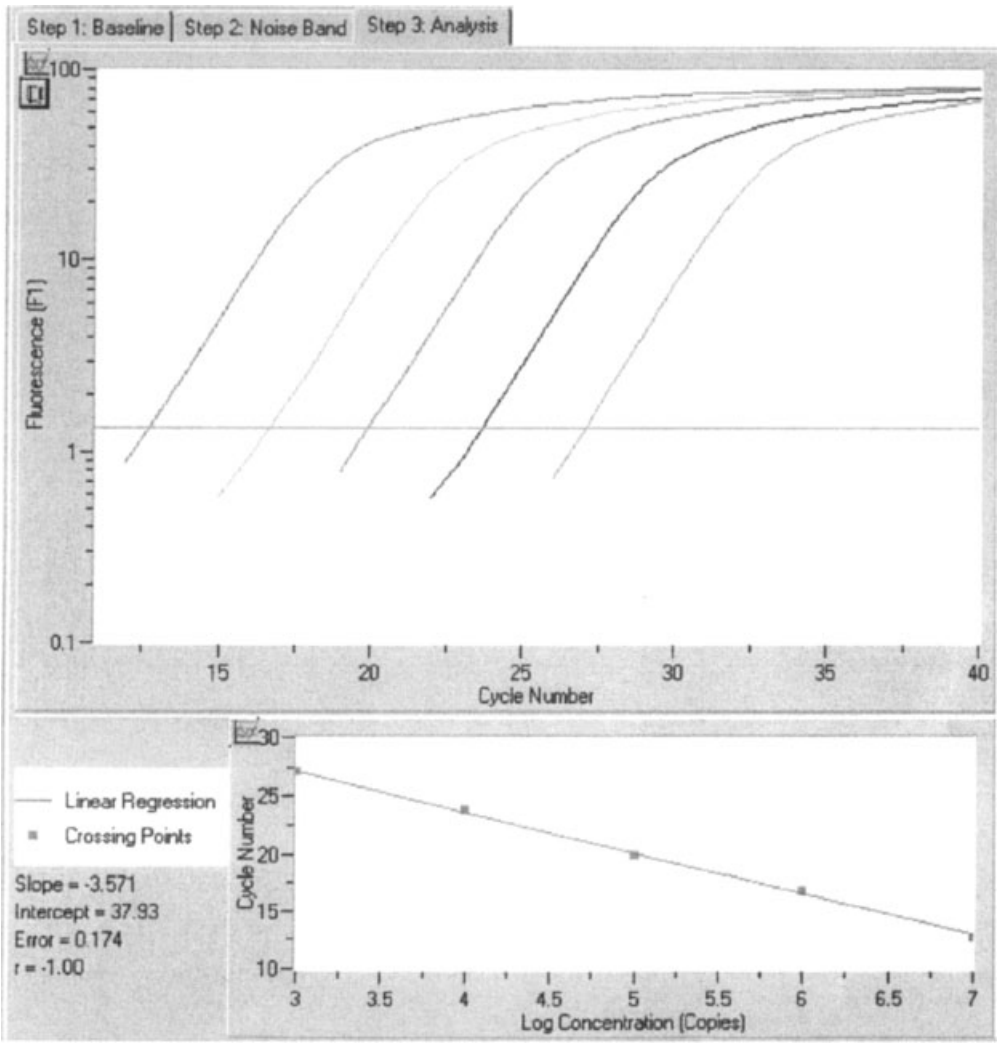

Fig. 1. Quantification of *KLK5* gene expression by real-time PCR. **Top**: A logarithmic plot of fluorescence signal above the noise level (horizontal line) during amplification. Serial dilutions of a total RNA preparation from prostate tissue were made and an arbitrary copy number was assigned to each sample according to the dilution factor. **Bottom**: The crossing points (cycle number), plotted against the log of copy number to obtain a standard curve. For details, see text.

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 Mc Nemar test and Wilcoxon signed ranks test. The binomial distribution was used to compute the significance level of the Mc Nemar 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 ± SD (standard deviation) of 1.70 ± 1.88 and from 0 to 3.67 in cancer tissues, with a mean ± SD of 0.65 ± 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.

TABLE I. *KLK5* Expression in Pairs of Non-Cancerous and Cancerous ProstaticTissues

KLK5 expression	No. of patients (%)	*P*-value*
Higher in normal vs. cancer	19 (66)	0.007
Lower in normal vs. cancer	5 (17)	
Approx. equal in both tissues	5 (17)	

*Calculated by McNemar non-parametric test.

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 II. *KLK5* Expression in Non-Cancerous and Cancerous Prostatic Tissues From 29 Patients

	Mean[a]	Standard deviation[a]	Median[a]	Range[a]	*P*-value[b]
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[c]	62%	—	83%		

[a]All values are expressed as ratios of *KLK5* and actin concentration, as described under Materials and Methods.
[b]Calculated by the Wilcoxon signed ranks test.
[c]Calculated by assuming that value in non-cancerous tissue is 100%.

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.

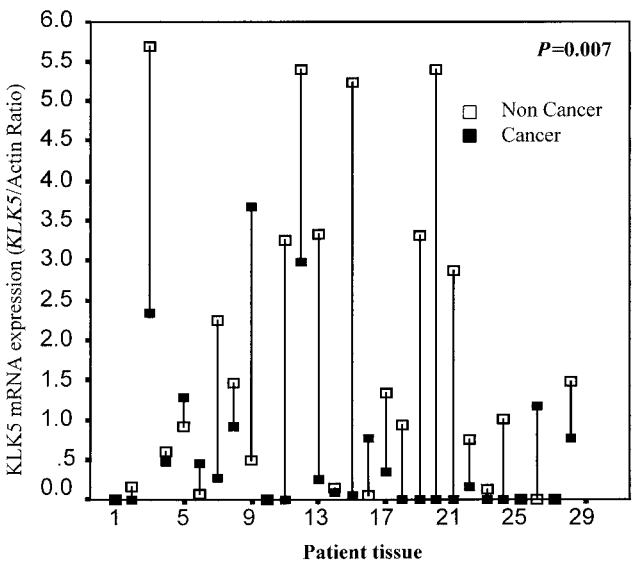

Fig. 2. Paired values of *KLK5* mRNA expression in non-cancer (□) and cancer (■) tissues for each patient. *P*-value was calculated by the McNemar non-parametric test.

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

	Total	Mean[a]	Standard deviation[a]	Median[a]	*P*-value[b]
Stage					
T1/T2	20	0.93	1.18	0.46	
T3	9	0.14	0.25	0.00	0.074
Gleason score					
2–4	13	0.98	1.11	0.47	
5–7	13	0.47	0.99	0.10	0.10
Grade					
G1/2	22	0.67	0.94	0.30	0.59
G3	7	0.68	1.46	0.08	

[a]mRNA levels (arbitrary units).
[b]Calculated by the Mann–Whitney U Test.

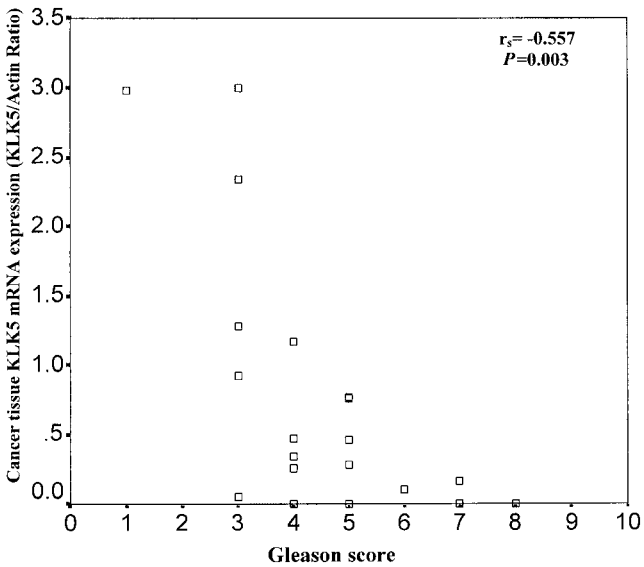

Fig. 3. Correlation between *KLK5* mRNA expression and Gleason score; r_s, Spearman correlation coefficient.

negative correlation between *KLK5* expression and the Gleason score. *KLK5* should be further studied as a potential prognostic marker for prostate cancer.

REFERENCES

1. Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. CA Cancer J 1999;49:8–31.
2. Diamandis EP. Prostate-specific antigen—its usefulness in clinical medicine. Trends Endocrinol Metab 1998;9:310–316.
3. Smith RA, Von Eschenbach AC, Wender A. American cancer society guidelines for early detection of cancer: update of early detection guidelines for prostate, colorectal and endometrial cancers, and update 2001: testing for early lung cancer detection. CA Cancer J Clin 2001;51:7–38.
4. Polascik TJ, Oesterling JE, Partin AW. Prostate specific antigen: a decade of discovery—what we have learned and where we are going. J Urol 1999;162:293–306.
5. Christiano A, Yoshida B, Dubauskas Z, Sokoloff M, Rinker-Schaeffer C. Development of markers of prostate cancer metastasis, review, and prospective. Urol Oncol 2000;5:217–223.
6. Abate-Shen C, Shen MM. Molecular genetics of prostate cancer. Genes Dev 2000;14:2410–2434.
7. Diamandis EP, Yousef GM, Clements J, Ashworth LK, Yoshida S, Egelrud T, Nelson PS, Shiosaka S, Little S, Lilja H, Stenman UH, Rittenhouse HG, Wain H. New nomenclature for the human tissue kallikrein gene family. Clin Chem 2000;46:1855–1858.
8. Yousef GM, Chang A, Scorilas A, Diamandis EP. Genomic organization of the human kallikrein gene family on chromosome 19q13.3–q13.4. Biochem Biophys Res Commun 2000;276:125–133.
9. Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV. The new human kallikrein gene family: implications in carcinogenesis. Trends Endocrinol Metab 2000;11:54–60.
10. Yousef GM, Diamandis EP. The new human tissue kallikrein gene family: structure, function and association to disease. Endocr Rev 2001;22:184–204.
11. Haese A, Becker C, Noldus J, Graefen M, Huland E, Huland H, Lilja H. Human glandular kallikrein 2: a potential serum marker for predicting the organ confirmed versus non-organ confined growth of prostate cancer. J Urol 2000;163:1491–1497.
12. Kwiatkowski MK, Recker F, Piironen T, Pettersson K, Otto T, Wernli M, Tscholl R. In prostatism patients the ratio of human glandular kallikrein to free PSA improves the discrimination between prostate cancer and benign hyperplasia within the diagnostic ''gray zone'' of total PSA 4 to 10 ng/ml. Urology 1998;52:360–365.
13. Magklara A, Scorilas A, Catalona WJ, Diamandis EP. The combination of human glandular kallikrein and free prostate-specific antigen (PSA) enhances discrimination between prostate cancer and benign prostatic hyperplasia in patients with moderately increased total PSA. Clin Chem 1999;45:1960–1966.
14. Rittenhouse HG, Finlay JA, Mikolajczyk SD, Partin AW. Human kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. Crit Rev Clin Lab Sci 1998;35:275–368.
15. Nelson PS, Gan L, Ferguson C, Moss P, Gelinas R, Hood L, Wang K. Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression. Proc Natl Acad Sci USA 1999;96:3114–3119.
16. Yousef GM, Obiezu CV, Luo LY, Black MH, Diamandis EP. Prostase/KLK-L1 is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues, and is hormonally regulated. Cancer Res 1999;59:4252–4256.
17. Yousef GM, Diamandis EP. The new kallikrein-like gene, KLK-L2. Molecular characterization, mapping, tissue expression, and hormonal regulation. J Biol Chem 1999;274:37511–37516.
18. Brattsand M, Egelrud T. Purification, molecular cloning, and expression of a human stratum corneum trypsin-like serine protease with possible function in desquamation. J Biol Chem 1999;274:30033–30040.
19. Kim H, Scorilas A, Katsaros D, Massobrio M, Yousef GM, Fracchioli S, Piccinno R, Gordini G, Diamandis EP. Human kallikrein gene 5 (KLK5) expression is an indicator of poor prognosis in ovarian cancer. Br J Cancer 2001;84:643–650.
20. Meyer A, Jung K, Lein M, Rudolph B, Schnorr D, Loening SA. Factors influencing the ratio of free to total prostate-specific antigen in serum. Int J Cancer 1997;74:630–636.
21. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 1997;22:130–131.
22. Bieche I, Onody P, Laurendeau I, Olivi M, Vidaud D, Lidereau R, Vidaud M. Real-time reverse transcription-PCR assay for future management of ERBB2-based clinical applications. Clin Chem 1999;45:1148–1156.
23. Bieche I, Olivi M, Champeme MH, Vidaud D, Lidereau R, Vidaud M. Novel approach to quantitative polymerase chain reaction using real-time detection: application to the detection of gene amplification in breast cancer. Int J Cancer 1998;78:661–666.
24. Woo TH, Patel BK, Cinco M, Smythe LD, Symonds ML, Norris MA, Dohnt MF. Real-time homogeneous assay of rapid cycle polymerase chain reaction product for identification of Leptonema illini. Anal Biochem 1998;259:112–117.
25. Henderson BE, Feigelson HS. Hormonal carcinogenesis. Carcinogenesis 2000;21:427–433.

26. Magklara A, Scorilas A, Stephan C, Kristiansen GO, Hauptmann S, Jung K, Diamandis EP. Decreased concentrations of prostate-specific antigen and human glandular kallikrein 2 in malignant versus nonmalignant prostatic tissue. Urology 2000; 56:527–532.
27. Diamandis EP. Prostate-specific antigen: a cancer fighter and a valuable messenger? Clin Chem 2000;46:896–900.
28. Guise TA. Parathyroid hormone-related protein and bone metastases. Cancer 1997;80:1572–1580.
29. Mundy GR. Mechanisms of bone metastasis. Cancer 1997;80: 1546–1556.
30. Termine JD, Belcourt AB, Christner PJ, Conn KM, Nylen MU. Properties of dissociatively extracted fetal tooth matrix proteins. I. Principal molecular species in developing bovine enamel. J Biol Chem 1980;255:9760–9768.
31. Lai LC, Erbas H, Lennard TW, Peaston RT. Prostate-specific antigen in breast cyst fluid: possible role of prostate-specific antigen in hormone-dependent breast cancer. Int J Cancer 1996; 66:743–746.
32. Fortier AH, Nelson BJ, Grella DK, Holaday JW. Antiangiogenic activity of prostate-specific antigen. J Natl Cancer Inst 1999;91: 1635–1640.