Association of transcript levels of 10 established or candidate-biomarker gene targets with cancerous versus non-cancerous prostate tissue from radical prostatectomy specimens

Vaananen, Riina-Minna; Lilja, Hans; Cronin, Angel; Kauko, Leni; Rissanen, Maria; Kauko, Otto; Kekki, Henna; Vidback, Siina; Nurmi, Martti; Alanen, Kalle; Pettersson, Kim

Published in:
Clinical Biochemistry

DOI:
10.1016/j.clinbiochem.2013.01.019

2013

Association of transcript levels of 10 established or candidate-biomarker gene targets with cancerous versus non-cancerous prostate tissue from radical prostatectomy specimens

Riina-Minna Väänänen[a]*, *Hans Lilja*[b,c,d,e], *Angel Cronin*[f], *Leni Kauko*[a], *Maria Rissanen*[a], *Otto Kauko*[a], *Henna Kekki*[a], *Siina Vidbäck*[a], *Martti Nurmi*[g], *Kalle Alanen*[h], *Kim Pettersson*[a]

[a] Division of Biotechnology, University of Turku, Turku, Finland

[b] Departments of Laboratory Medicine, Surgery (Urology), and Medicine (GU-Oncology), Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.

[c] Nuffield Department of Surgical Sciences, University of Oxford, Oxford, United Kingdom

[d] Institute of Biomedical Technology, University of Tampere, Tampere, Finland

[e] Department of Laboratory Medicine, Lund University, Skåne University Hospital, Malmö, Sweden

[f] Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.

[g] Department of Surgery, Turku University Hospital, Turku, Finland

[h] Department of Pathology, Turku University Hospital, Turku, Finland

*Corresponding author: Division of Biotechnology, University of Turku, Tykistökatu 6A 6th floor, 20520 Turku, Finland. Tel +358 2 333 8905 Fax +358 2 333 8050 Email: riina-minna.vaananen@utu.fi

ABSTRACT

OBJECTIVES: The benefits of PSA (prostate specific antigen)-testing in prostate cancer remain controversial with a consequential need for validation of additional biomarkers. We used highly standardized reverse-transcription (RT)-PCR assays to compare transcript levels of 10 candidate cancer marker genes – *BMP6*, *FGF-8b*, *KLK2*, *KLK3*, *KLK4*, *KLK15*, *MSMB*, *PCA3*, *PSCA* and *Trpm8* – in carefully ascertained non-cancerous versus cancerous prostate tissue from patients with clinically localized prostate cancer treated by radical prostatectomy.

DESIGN AND METHODS: Total RNA was isolated from fresh frozen prostate tissue procured immediately after resection from two separate areas in each of 87 radical prostatectomy specimens. Subsequent histopathological assessment classified 86 samples as cancerous and 88 as histologically benign prostate tissue. Variation in total RNA recovery was accounted for by using external and internal standards and enabled us to measure transcript levels by RT-PCR in a highly quantitative manner.

RESULTS: Of the ten genes, there were significantly higher levels only of one of the less abundant transcripts, *PCA3*, in cancerous versus non-cancerous prostate tissue whereas *PSCA* mRNA levels were significantly lower in cancerous versus histologically benign tissue. Advanced pathologic stage was associated with significantly higher expression of *KLK15* and *PCA3* mRNAs. Median transcript levels of the most abundantly expressed genes (i.e. *MSMB*, *KLK3*, *KLK4* and *KLK2*) in prostate tissue were up to 10^5-fold higher than those of other gene targets.

CONCLUSIONS: *PCA3* expression was associated with advanced pathological stage but the magnitude of overexpression of *PCA3* in cancerous versus non-cancerous prostate tissue was modest compared to previously reported data.

Keywords: Prostatic Neoplasms, Reverse Transcriptase Polymerase Chain Reaction

1. INTRODUCTION

Although levels of prostate-specific antigen (PSA) in blood are strongly associated with risk and outcome of prostate cancer (PCa) [1] and PSA-based screening can reduce PCa mortality, current screening algorithms lead to overdetection and consequential overtreatment of PCa [2]. Hence, the search continues for novel biomarkers that could contribute to the diagnostic information above and beyond that provided by the widely used conventional testing for total PSA levels in the blood. Nucleic acid-based gene expression signatures have been suggested to aid in the prediction of unfavourable PCa compared to the established clinical predictors [3].

We studied the gene expression levels of 10 nucleic acid markers assessing their suitability for discriminating cancerous tissue of different pathological stage and grade from non-cancerous tissue. Each of these markers has previously been suggested to contribute diagnostic or predictive value in PCa diagnostics. *PCA3* (prostate cancer antigen 3), discovered in 1999 [4], has recently been launched as a commercial application for the detection of increased mRNA expression in prostate cells shed into urine, and is claimed to improve the inadequacies of PSA-based diagnosing of PCa [5]. *BMP6* (bone morphogenetic protein 6) [6], *FGF-8b* (fibroblast growth factor 8, isoform b) [7], *KLK2* (kallikrein-related peptidase 2) [8], *KLK4* (kallikrein-related peptidase 4) [9], *KLK15* (kallikrein-related peptidase 15) [10], *PSCA* (prostate stem cell antigen) [11] and *Trpm8* (transient receptor potential protein 8) [12] have been reported to be overexpressed in PCa whereas the expression of *KLK3* (kallikrein-related peptidase 3 encoding the PSA protein) [8] and *MSMB* (microseminoprotein-beta) has been reported to be decreased in cancerous prostate tissue [13].

The study was performed by analyzing prostate tissue samples from 87 PCa patients with a truly quantitative, internally standardized real-time reverse transcription-PCR (RT-PCR) assay

technique, described previously [14]. Based on traditional RT-PCR methods, data are often only qualitative or semi-quantitative, and reliable quantitative information is difficult, if not impossible, to achieve with gel-based techniques or by relative quantification using house-keeping genes. The unavoidable loss in recovery during the several steps of RT-PCR protocol such as RNA extraction and reverse transcription may also lead to underestimation of the original levels of mRNA. These problematic aspects were taken into account in our work, where closed-tube real-time detection of the PCR amplification product by time-resolved fluorometry was designed to increase the robustness and sensitivity of the assay. Additionally, an artificial internal standard was used to bypass the need to measure reference gene expression and to correct for the inherent variation in mRNA recovery. To our knowledge, this is the first study to truly and reliably quantify the mRNA levels of these target genes in a prostate tissue cohort.

2. MATERIALS AND METHODS

2.1 Patient cohort and sample collection

Tissue samples were obtained from 87 PCa patients immediately after radical prostatectomy. From each prostate, a tissue sample wedge was obtained from the suspected cancerous area and another from an assumed control area within minutes from the surgical removal of the prostate (Supplemental Figure 1). A small tissue specimen size ensured the best possible homogeneity of material. Based on the histological examination of the immediately adjacent tissue surrounding the sampling site, 76 samples were classified as histologically benign tissue, 12 as prostatic intraepithelial neoplasia (PIN) tissue, and 86 as cancerous tissue ranging from Gleason grade 2 to 5. The estimated median proportion of cancerous tissue was 30% (interquartile range: 10, 55) among the cancerous samples. In subsequent analyses, the PIN samples were considered as histologically

benign tissue. Patient characteristics are shown in Supplemental Table 1. The study protocol was approved by the local Ethics committee and it was in accordance with the Helsinki Declaration of 1975, as revised in 1996, with written informed consent obtained from each participant.

2.2 RNA extraction and cDNA synthesis

Tissue samples were collected and processed as described previously [15]. A known amount of internal RNA standard - RNA from an artificial, mutant form of *KLK3* gene called mmPSA [16] - was added to each sample after cell lysis.

2.3 Real-time PCR

Time-resolved fluorometry, including lanthanide chelate-labeled probes that specifically recognize amplified targets [14], was utilized for the real-time detection of the PCR amplification. The oligonucleotides were purchased from Thermo Fisher Scientific (Germany) and MWG (Germany) (Supplemental Table 2). The lanthanide probes were designed, labelled and purified in-house [19, 20]. PCR conditions for each assay are described in Supplemental Table 3. Samples were run in triplicate reactions and the thermocycling was performed as described previously [15] with HotMaster™ Taq DNA Polymerase (Eppendorf, Hamburg, Germany) or AmpliTaq® Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA) with the corresponding buffers. Specific purified PCR products were produced as described previously [15] and used as external standards in concentrations that are presented in Supplemental Table 4.

2.4 Data analysis

The samples were considered positive only if all three PCR replicates were above the limit of detection (LOD, defined as the lowest standard which produces a rise in fluorescence signal) and

the technical negative controls did not produce a rise in the signals. The original copy numbers were calculated according to internal standard values and normalized to total RNA amount [15].

Statistical analyses were performed with Stata 10.0 (StataCorp LP, College Station) at the Department of Epidemiology and Biostatistics, Memorial Sloan-Kettering Cancer Center (New York, NY, USA). To evaluate the association between each gene and the outcome measures, univariate logistic regression was used with the cluster option to account for multiple samples per patient. For *FGF-8b* mRNA, samples were categorized as negative (non-detectable expression) or positive (detectable expression), since many specimens had mRNA levels equal to the LOD of the assay.

3. RESULTS

3.1 Expression of the 10 gene targets

The frequencies of detectable expression of the 10 gene targets are shown in Table 1. The expression levels of studied mRNAs ranged over 2-4 orders of magnitude with considerable overlaps between histologically benign and cancerous samples. The median expression levels for highest expressed mRNAs (*MSMB*, *KLK3*, *KLK4* and *KLK2*) were $6.8x10^9$, $2.1x10^9$, $1.3x10^9$ and $3.5x10^8$ mRNA copies/µg of total RNA in cancerous group and $1.1x10^{10}$, $2.7x10^9$, $1.5x10^9$ and $4.6x10^8$ mRNA copies/µg of total RNA in the group of histologically benign samples. The lowest expression levels were found with *KLK15* and *FGF-8b* mRNAs. Median expression level for *KLK15* was $1.2x10^5$ mRNA copies/µg of total RNA in the cancerous group and $8.8x10^4$ mRNA copies/µg total RNA in the group of histologically benign samples. *FGF-8b* mRNA expression was detectable in only 33% of the cancerous samples and in 28% of the histologically benign group, but

within the positive samples, the median values were $1.6x10^2$ mRNA copies/µg of total RNA in the cancerous samples and $1.8x10^2$ mRNA copies/µg of total RNA in the group of histologically benign samples.

Of the 10 mRNA targets, only the expression levels of *PCA3* and *PSCA* were significantly different ($p<0.05$) in cancerous than histologically benign tissue. The cancerous tissue samples had significantly higher levels of *PCA3* mRNA (median 5.75-fold difference) than the histologically benign samples whereas *PSCA* mRNA levels were significantly lower in cancerous tissue (median 2.9-fold difference) (Figure 1, Table 2). Among the 46 patients that provided one cancerous and one benign sample with detectable *PCA3* mRNA expression, the median *PCA3* mRNA ratio between the cancerous and normal sample was 2.9 with an interquartile range of 0.68−15 and with 10th and 90th percentiles at 0.24 and 638, respectively. In 31/46 patients, the cancerous sample had higher *PCA3* mRNA expression. There were also the same number of patients (n=46) providing one cancerous and one benign sample with detectable *PSCA* mRNA expression. Among that group, *PSCA* mRNA levels were lower in the cancerous sample for 25/46 patients and the median *PSCA* mRNA ratio between the histologically benign and cancerous sample was 1.1 with an interquartile range of 0.51-3.6 and with 10^{th} and 90^{th} percentiles at 0.07 and 9.8, respectively.

ROC curve analysis of *PCA3* and *PSCA* mRNA expression in cancerous and histologically benign prostatic tissues produced AUC values of 0.673 for *PCA3* and 0.414 for *PSCA* (Figure 2).

3.2 Association of gene expression with Gleason grade and pathologic stage

Of the 10 studied mRNA targets, only the expression of *KLK3* mRNA was significantly associated with Gleason grade ($p=0.018$) with higher levels in cancerous tissue of grade ≤ 3 than in grade ≥ 4 (Table 3). Advanced pathologic stage was significantly associated with higher levels of *KLK15*

mRNA (p=0.032) and *PCA3* mRNA (p=0.015) (Table 4). None of the other eight mRNAs were significantly associated with the pathologic stage.

3.3 Effect of cancer cell percentage on the levels of gene expression

We also analysed samples containing either less or more than 50% cancerous tissue as separate groups, but the cancer cell percentage in the tissue specimens did not have a significant impact on the mRNA levels (Supplemental Table 5).

4. DISCUSSION

Despite continued controversy on PSA testing and a widely acknowledged need of novel markers predicting PCa risk or outcome, it does not appear likely that any novel marker will single-handedly prove to be more widely useful than PSA. In this study, we determined quantitatively the mRNA levels of suggested PCa marker genes in cancerous and non-cancerous tissue samples freshly obtained from prostatectomy specimens to nominate a subset of the markers in this panel that hold potential promise for distinguishing cancerous prostate tissue from non-cancerous tissue. Each of these genes has previously been described in the literature but our study is unique in reporting the level of gene expression as absolute mRNA copy numbers determined by quantitative, internally standardized real-time RT-PCR assays. By utilizing an artificial internal standard as has been recommended [23] but rarely used, our approach provides accurate data and bypasses the need and pitfalls of measuring any reference gene expression.

We found the median expression levels of the most abundantly expressed genes - *MSMB*, *KLK3*, *KLK4* and *KLK2* - to be up to 10^5-fold higher than the mRNA transcript levels of the gene targets

with the lowest expression levels but none of them were significantly differently expressed in cancerous versus non-cancerous tissue. Only the expression of two of the less abundant target mRNAs – *PCA3* and *PSCA* - was significantly different in the cancerous tissue compared to the histologically benign tissue despite the considerable overlap in the expression of all 10 genes between cancerous and histologically benign tissue. *PCA3* was the marker that provided the best discrimination between the groups. However, the magnitude of *PCA3* overexpression in cancerous compared to the non-cancerous tissue was smaller in this cohort than in prior reports. In the first and original *PCA3* study by Bussemakers et al a 10–100-fold overexpression was observed using Northern blot [4] and later on Hessels et al found an 11- or 66-fold difference between cancerous and normal tissues from cancer-affected prostates depending on whether the cancerous samples contained less or more than 10% cancer cells [24]. However, we did not find any direct correlation between the cancer cell percentage and levels of *PCA3* mRNA or any of the target mRNAs in this panel. The level of *PCA3* overexpression has been reported to vary also in other studies [21, 25] and our observation of a 5.75-fold upregulation, detected by RT-PCR, is at the lower end of the range. Recently Robert et al [25] described a 30-fold upregulation in cancerous tissue compared to normal areas of cancer-affected prostates, but they also noted that the level of *PCA3* overexpression was further increased when cancerous tissue was compared to BPH tissue, and they suggested that upregulation of *PCA3* expression may start early on in the disease progression before the cells turn truly cancerous. That can be described as a cancer field effect which causes molecular level alterations in histologically benign-appearing tissue. Other *PCA3*-related observations of field effect in prostate cancer have also been reported [26] and the cohort studied in our work could conceivably present similar field effect–related changes in mRNA expression. Magnitude of *PSCA* underexpression was moderate as well and underexpression in cancerous tissue is contrary to the earlier reports of *PSCA* overexpression in PCa [11]. A limitation of this study, considering the

likelihood of a significant field effect, is the lack of control tissues from non-PCa patients such as those that were included in the recent study of Robert et al [25]. Such a study is in progress.

Gleason grade did not associate with the mRNA copy numbers apart from the modestly lower expression of *KLK3* mRNA at a higher tumor grade. However, an advanced pathologic stage was associated with the overexpression of *KLK15* and *PCA3* mRNAs. This is supported by the association between *KLK15* levels and Gleason grade in prostate tissue that was previously reported by Stephan et al [10], and adds *KLK15* to the group of most interesting markers of this panel but further studies are needed to find out the potential connection between these markers and prognosis. In this cohort, the follow-up times were too short (<3 years) to justify any prognostic analyses.

5. CONCLUSIONS

Of the 10 target genes, *PCA3* and *PSCA* mRNAs were significantly differently expressed in cancerous than in histologically benign tissue of cancerous prostates and *KLK15* mRNA in more advanced cancer stage. However, the differences in *PCA3* expression in particular were not as pronounced as previously has been reported, leading us to assume indications of field effect in the non-cancerous tissue of the cancerous prostates. To obtain a better view on the true roles of these 10 RNA markers in prostate carcinogenesis, we suggest further studies especially regarding the best-performing markers identified in this study.

6. DISCLOSURE STATEMENT

None of the authors declare any conflict of interest regarding this paper except for Dr. Hans Lilja who holds patents for free PSA, intact PSA, and hK2 assays.

7. ROLE OF THE FUNDING SOURCE

This study was supported by Academy of Finland (Project 206690), European Union 6th Framework contract LSHC-CT-2004-503011 (P-Mark), National Cancer Institute (grant number P50-CA92629), Swedish Cancer Society (grant number 0345), Swedish Research Council (Medicine) (grant number 20095), the Sidney Kimmel Center for Prostate and Urologic Cancers, and David H. Koch through the Prostate Cancer Foundation. The sponsors had no role in study design; in the collection, analysis, or interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

REFERENCES

[1] I.M. Thompson, D.K. Pauler, P.J. Goodman, C.M. Tangen, M.S. Lucia, H.L. Parnes et al, Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter, N Engl J Med. 350 (2004) 2239–46.

[2] F.H. Schröder, J. Hugosson, M.J. Roobol, T.L. Tammela, S. Ciatto, V. Nelen et al, Screening and prostate-cancer mortality in a randomized European study, N Engl J Med. 360 (2009) 1320–78.

[3] A.J. Stephenson, A. Smith, M.W. Kattan, J. Satagopan, V.E. Reuter, P.T. Scardino et al, Integration of gene expression profiling and clinical variables to predict prostate carcinoma recurrence after radical prostatectomy, Cancer. 104 (2005) 290–8.

[4] M.J. Bussemakers, A. van Bokhoven, G.W. Verhaegh, F.P. Smit, H.F. Karthaus, J.A. Schalken et al, DD3: a new prostate-specific gene, highly overexpressed in prostate cancer, Cancer Res. 59 (1999) 5975–9.

[5] J. Groskopf, S.M. Aubin, I.L. Deras, A. Blase, S. Bodrug, C. Clark et al, APTIMA PCA3 molecular urine test: development of a method to aid in the diagnosis of prostate cancer, Clin Chem. 52 (2006) 1089-95.

[6] J. Barnes, C.T. Anthony, N. Wall, M.S. Steiner, Bone morphogenetic protein-6 expression in normal and malignant prostate, World J Urol. 13 (1995) 337–43.

[7] T.J. Dorkin, M.C. Robinson, C. Marsh, A. Bjartell, D.E. Neal, H.Y. Leung, FGF8 over-expression in prostate cancer is associated with decreased patient survival and persists in androgen independent disease, Oncogene. 18 (1999) 2755–61.

[8] M.F. Darson, A. Pacelli, P. Roche, H.G. Rittenhouse, R.L. Wolfert, C.Y. Young et al, Human glandular kallikrein 2 (hK2) expression in prostatic intraepithelial neoplasia and adenocarcinoma: a novel prostate cancer marker, Urology. 49 (1997) 857–62.

[9] Z. Xi, T.I. Klokk, K. Korkmaz, P. Kurys, C. Elbi, B. Risberg et al, Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer, Cancer Res. 64 (2004) 2365–70.

[10] C. Stephan, G.M. Yousef, A. Scorilas, K. Jung, M. Jung, G. Kristiansen et al, Quantitative analysis of kallikrein 15 gene expression in prostate tissue, J Urol. 169 (2003) 361–4.

[11] R.E. Reiter, Z. Gu, T. Watabe, G. Thomas, K. Szigeti, E. Davis et al, Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer, Proc Natl Acad Sci U S A. 95 (1998) 1735–40.

[12] L. Tsavaler, M.H. Shapero, S. Morkowski, R. Laus, Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins, Cancer Res. 61 (2001) 3760–9.

[13] T. Tsurusaki, T. Koji, H. Sakai, H. Kanetake, P.K. Nakane, Y. Saito, Cellular expression of beta-microseminoprotein (beta-MSP) mRNA and its protein in untreated prostate cancer, Prostate. 35 (1998) 109–116.

[14] J. Nurmi, T. Wikman, M. Karp, T. Lövgren, High-performance real-time quantitative RT-PCR using lanthanide probes and a dual-temperature hybridization assay, Anal Chem. 74 (2002) 3525–32.

[15] R.-M. Väänänen, M. Rissanen, O. Kauko, S. Junnila, V. Väisänen, J. Nurmi et al, Quantitative real-time RT-PCR assay for PCA3, Clin Biochem. 41 (2008) 103–8.

[16] J. Nurmi, H. Lilja, A. Ylikoski, Time-resolved fluorometry in end-point and real-time PCR quantification of nucleic acids, Luminescence. 15 (2000) 381–8.

[17] M.P. Valta, J. Tuomela, H. Vuorikoski, N. Loponen, R.-M. Väänänen, K. Pettersson et al, FGF-8b induces growth and rich vascularization in an orthotopic PC-3 model of prostate cancer, J Cell Biochem. 107 (2009) 769–84.

[18] A. Ylikoski, M. Karp, K. Pettersson, H. Lilja, T. Lövgren, Simultaneous quantification of human glandular kallikrein 2 and prostate-specific antigen mRNAs in peripheral blood from prostate cancer patients, J Mol Diagn. 3 (2001) 111–22.

[19] M. Rissanen, P. Helo, R.-M. Väänänen, V. Wahlroos, H. Lilja, M. Nurmi et al, Novel homogenous time-resolved fluorometric RT-PCR assays for quantification of PSA and hK2 mRNAs in blood, Clin Biochem. 40 (2007) 111–8.

[20] J. Nurmi, A. Ylikoski, T. Soukka, M. Karp, T. Lövgren, A new label technology for the detection of specific polymerase chain reaction products in a closed tube, Nucleic Acids Res. 28 (2000):E28.

[21] J.B. de Kok, G.W. Verhaegh, R.W. Roelofs, D. Hessels, L.A. Kiemeney, T.W. Aalders et al, DD3(PCA3), a very sensitive and specific marker to detect prostate tumors, Cancer Res. 62 (2002) 2695–8.

[22] P. Helo, A.M. Cronin, D.C. Danila, S. Wenske, R. Gonzalez-Espinoza, A. Anand et al, Circulating prostate tumor cells detected by reverse transcription-PCR in men with localized or castration-refractory prostate cancer: concordance with CellSearch assay and association with bone metastases and with survival, Clin Chem. 55 (2009) 765–73.

[23] J. Huggett, K. Dheda, S. Bustin, A. Zumla. Real-time RT-PCR normalisation; strategies and considerations, Genes Immun. 6 (2005) 279–84.

[24] D. Hessels, J.M. Klein Gunnewiek, I. van Oort, H.F. Karthaus, G.J. van Leenders, B. van Balken et al, DD3(PCA3)-based molecular urine analysis for the diagnosis of prostate cancer, Eur Urol. 44 (2003) 8–15.

[25] G. Robert, S. Jannink, F. Smit, T. Aalders, D. Hessels, R. Cremers et al, Rational basis for the combination of PCA3 and TMPRSS2:ERG gene fusion for prostate cancer diagnosis, Prostate. (2012). doi: 10.1002/pros.22546.

[26] I. Popa, Y. Fradet, G. Beaudry, H. Hovington, G. Beaudry, B. Têtu, Identification of PCA3 (DD3) in prostatic carcinoma by in situ hybridization, Mod Pathol. 20 (2007) 1121-7.

FIGURE LEGENDS

Figure 1. Expression levels of *BMP6*, *KLK2*, *KLK3*, *KLK4*, *KLK15*, *MSMB*, *PCA3*, *PSCA* and *Trpm8* mRNAs in 88 non-cancerous and 86 cancerous prostate tissue samples. The boxes denote the interquartile ranges, the lines in the middle of the boxes show the median values and the whiskers denote the 10th and 90th percentiles.

Figure 2. ROC curve analysis of *PCA3* and *PSCA* mRNA expression in histologically benign prostatic tissues (n=88) vs cancerous prostatic tissues (n=86). AUC values are 0.673 for *PCA3* mRNA and 0.414 for *PSCA* mRNA.

Supplemental Figure 1. Flow chart of the sample handling process.

Figure 1.

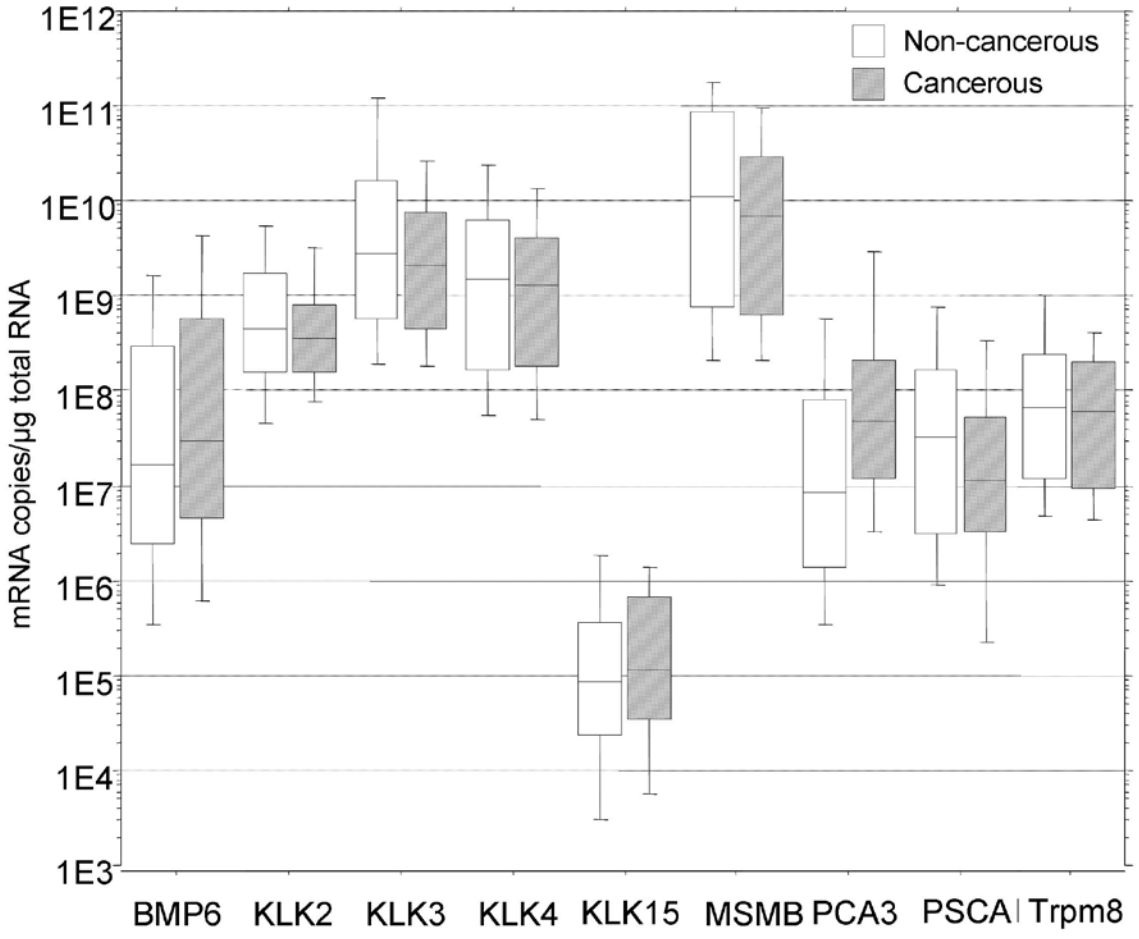
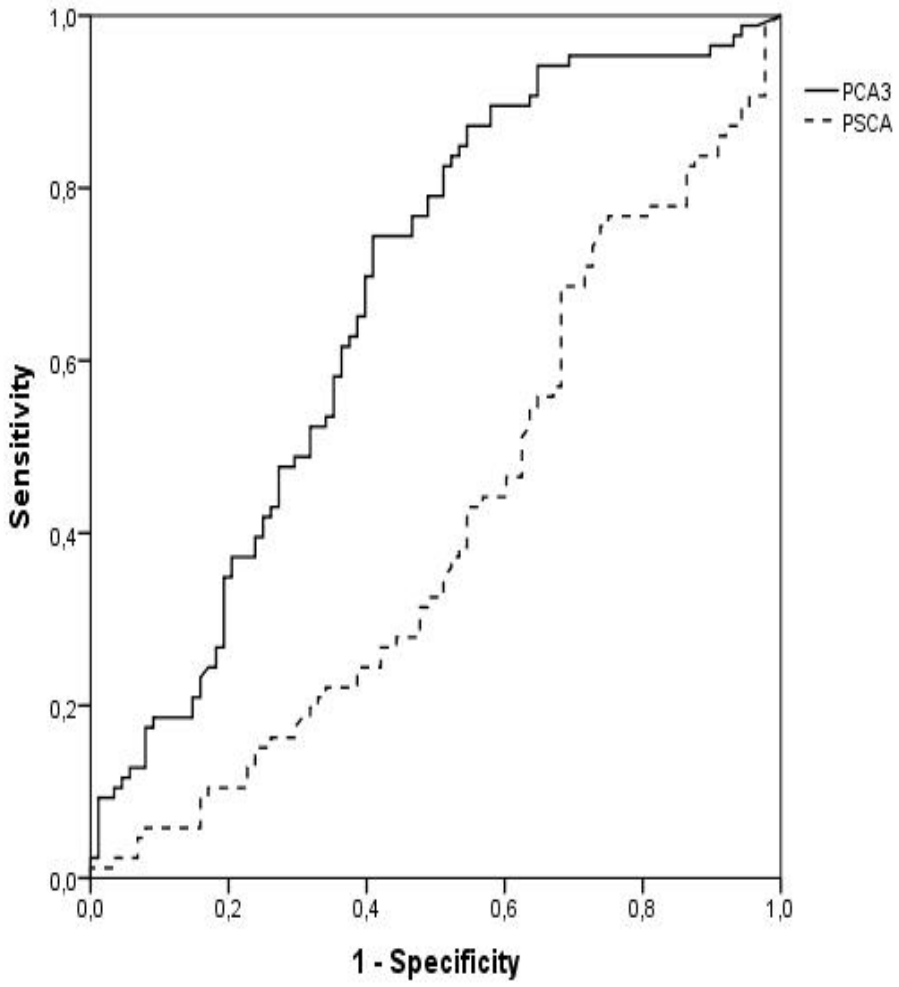

1E12
1E11
1E10
1E9
1E8
1E7
1E6
1E5
1E4
1E3
mRNA copies/µg total RNA
Non-cancerous
Cancerous
BMP6
KLK2
KLK3
KLK4
KLK15
MSMB
PCA3
PSCA
Trpm8

Figure 2.


1,0
0,8
0,6
0,4
0,2
0,0
Sensitivity
0,0
0,2
0,4
0,6
0,8
1,0
1 - Specificity
PCA3
PSCA

Supplemental Figure 1.

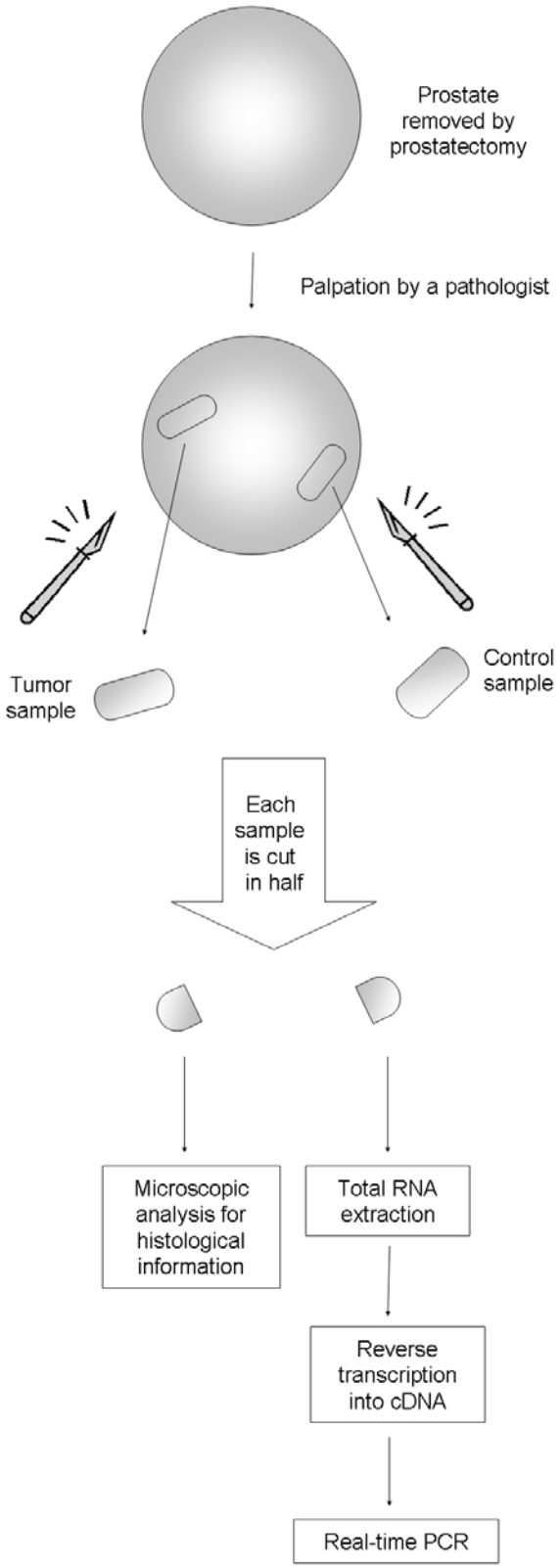


Table 1. Frequencies of detectable expression of 10 gene targets.

Target gene	Non-cancerous (n=88)	Cancerous (n=86)
BMP6	87 (99%)	85 (99%)
FGF-8b	25 (28%)	28 (33%)
KLK2	85 (97%)	85 (99%)
KLK3	86 (98%)	85 (99%)
KLK4	88 (100%)	86 (100%)
KLK15	83 (94%)	79 (92%)
MSMB	88 (100%)	86 (100%)
PCA3	85 (97%)	85 (99%)
PSCA	86 (98%)	85 (99%)
Trpm8	88 (100%)	85 (99%)

Number of positive samples is noted for each target mRNA.

Table 2. Association of gene expression with cancer status of the tissue sample.

Target gene	Non-cancerous (n=88) (log_{10}copies/µg total RNA)	Cancerous (n=86) (log_{10}copies/µg total RNA)	Fold difference in medians	Univariate P value
BMP6	7.23 (6.41, 8.53)	7.49 (6.68, 8.76)	1.81	0.16
FGF-8b > LOD	25 (28%)	28 (33%)	NA	0.5
KLK2	8.69 (8.25, 9.26)	8.55 (8.23, 8.91)	0.76	0.3
KLK3	9.46 (8.78, 10.2)	9.31 (8.68, 9.87)	0.75	0.3
KLK4	9.17 (8.23, 9.79)	9.10 (8.26, 9.61)	0.85	0.5
KLK15	5.00 (4.46, 5.65)	5.13 (4.72, 5.84)	1.32	0.5
MSMB	10.0 (8.87, 10.9)	9.83 (8.80, 10.5)	0.61	0.16
PCA3	7.00 (6.32, 7.92)	7.69 (7.22, 8.31)	5.75	<0.0005
PSCA	7.55 (6.57, 8.22)	7.06 (6.57, 7.72)	0.35	0.046
Trpm8	7.82 (7.07, 8.38)	7.78 (7.07, 8.29)	0.91	0.4

Data are given as median (interquartile range) or frequency (percentage). LOD, limit of detection; NA, not available. In statistical analyses samples were categorized as negative or positive for *FGF-8* mRNA. For the other genes, the rank of expression level was entered as the independent variable to account for their skewed distribution.

Table 3. Association of gene expression with Gleason grade of the tissue sample.

Target gene	Gleason grade 2 or 3 (n=62) (log_{10}copies/µg total RNA)	Gleason grade 4 or 5 (n=24) (log_{10}copies/µg total RNA)	Fold difference in medians	Univariate P value
BMP6	7.59 (6.78, 9.06)	7.20 (6.56, 8.22)	0.41	0.16
FGF-8b > LOD	19 (31%)	9 (38%)	NA	0.5
KLK2	8.61 (8.23, 8.96)	8.32 (8.17, 8.73)	0.52	0.19
KLK3	9.51 (8.84, 10.1)	8.89 (8.41, 9.69)	0.27	0.018
KLK4	9.20 (8.46, 9.68)	8.81 (7.82, 9.40)	0.41	0.08
KLK15	5.20 (4.81, 5.82)	5.06 (4.72, 5.88)	0.82	0.8
MSMB	9.91 (8.80, 10.5)	9.45 (8.64, 10.4)	0.35	0.4
PCA3	7.80 (7.25, 8.39)	7.56 (6.95, 7.99)	0.59	0.4
PSCA	7.07 (6.65, 7.81)	6.91 (6.00, 7.52)	0.74	0.2
Trpm8	7.86 (7.08, 8.35)	7.44 (6.78, 8.23)	0.39	0.2

Data are given as median (interquartile range) or frequency (percentage). LOD, limit of detection; NA, not available. In statistical analyses samples were categorized as negative or positive for *FGF-8b* mRNA. For the other genes, the rank of expression level was entered as the independent variable to account for their skewed distribution.

Table 4. Association of gene expression with pathologic stage of the prostate specimen.

Target gene	pT2 (n=72) (log_{10}copies/µg total RNA)	pT3/4 (n=88) (log_{10}copies/µg total RNA)	Fold difference in medians	Univariate P value
BMP6	7.08 (6.25, 7.96)	7.53 (6.65, 8.69)	2.63	0.096
FGF-8b > LOD	23 (32%)	26 (30%)	NA	0.8
KLK2	8.49 (8.13, 9.03)	8.62 (8.23, 9.09)	1.52	0.4
KLK3	9.04 (8.53, 9.90)	9.61 (8.83, 10.0)	3.98	0.08
KLK4	8.85 (8.02, 9.53)	9.28 (8.45, 9.82)	2.64	0.13
KLK15	4.80 (4.29, 5.51)	5.23 (4.79, 5.77)	2.71	0.032
MSMB	9.64 (8.69, 10.5)	9.94 (9.01, 10.7)	2.00	0.3
PCA3	7.16 (6.37, 7.95)	7.62 (6.91, 8.24)	3.71	0.015
PSCA	7.03 (6.46, 7.86)	7.23 (6.61, 8.04)	1.90	0.2
Trpm8	7.58 (6.82, 8.18)	7.89 (7.17, 8.34)	2.09	0.069

Data are given as median (interquartile range) or frequency (percentage). LOD, limit of detection; NA, not available. In statistical analyses samples were categorized as negative or positive for *FGF-8b* mRNA. For the other genes, the rank of expression level was entered as the independent variable to account for their skewed distribution.

Supplemental Table 1. Patient characteristics.

	Median (interquartile range) or frequency (percentage)
Number of patients	87
Age at surgery (years)	64 (58, 67)
Pathologic stage[1]	
pT2	36 (45%)
pT3/pT4	44 (55%)
Pathologic Gleason score[2]	
≤ 6	46 (57%)
7	22 (27%)
≥ 8	13 (16%)
Tissue samples analyzed	
2 non-cancerous	20 (23%)
1 non-cancerous, 1 cancerous	48 (55%)
2 cancerous	19 (22%)

[1]Pathologic stage was unknown for 7 patients.

[2]Pathologic Gleason score was unknown for 6 patients.

Supplemental Table 2. Oligonucleotides used in this study.

Oligonucleotide	Sequence	Location	Database sequence	Reference
BMP6 5’ primer	5’-GCA TCA TCA GCA CAG AGA CTC TGA C-3’	1025-1046	NM001718	
BMP6 3’ primer	5’-ACG TGG ACT CCA TCC CTT G-3’	1177-1195	NM001718	
BMP6 reporter probe	5’-Ln-GGC TGG AAT TTG ACA TCA CGG CCT G-3’	1093-1117	NM001718	
BMP6 quencher probe	5’-TCA AAT TCC AGC C-3’	1093-1105	NM001718	
FGF-8b 5’ primer				(17)
FGF-8b 3’ primer				(17)
FGF-8b reporter probe				(17)
FGF-8b quencher probe				(17)
KLK2 5’ primer				(18)
KLK2 3’ primer				(18)
KLK2 reporter probe				(19)
KLK2 quencher probe				(19)
KLK3 5’ primer				(20)
KLK3 3’ primer				(20)
KLK3 reporter probe				(20)
KLK3 quencher probe				(14)
KLK4 5’ primer	5’-GGC ACT GGT CAT GGA AAA CG-3’	138-157	NM_004917	
KLK4 3’ primer	5’-CAG CCC GAT GGT GTA GGA GTT-3’	223-243	NM_004917	
KLK4 reporter probe	5’-Ln-CAG CAC CCA CTG CGG ATG CAC CAG-3’	178-201	NM_004917	
KLK4 quencher probe	5’-CCG CAG TGG GTG CTG-Q-3’	187-201	NM_004917	
KLK15 5’ primer	5’-CTG CCA AAG CCG CTT C-3’	186-201	AY373373	
KLK15 3’ primer	5’-ATG ACC CGA GAC GTG G-3’	263-278	AY373373	
KLK15 reporter probe	5’-Ln-CGC CTG GGA GAG CAC AAC CTG-3’	211-231	AY373373	
KLK15 quencher probe	5’-CTC TCC CAG GCG-3’	211-223	AY373373	
mmPSA 5’ primer				(16)
mmPSA 3’ primer				(16)
mmPSA reporter probe				(20)
mmPSA quencher probe				(14)
MSMB 5’ primer	5’-CCT GCT TAT CAC AAT GAA TGT TC-3’	20-42	NM_002443	
MSMB 3’ primer	5’-CAT TCA CTG ACA GAA CAG GTC-3’	347-367	NM_002443	
MSMB reporter probe	5’-Ln-TCC TTC TTC TCC ACC ACG ATA TAC TTG C-3’	310-337	NM_002443	
MSMB quencher probe	5’-GTG GAG AAG AAG GA-3’	324-337	NM_002443	
PCA3 5’ primer				(21)
PCA3 3’ primer				(21)
PCA3 reporter probe				(21)
PCA3 quencher probe				(15)
PSCA 5’ primer				(22)

PSCA 3’ primer				(22)
PSCA reporter probe				(22)
PSCA quencher probe				(22)
Trpm8 5’ primer	5’-GAG TTG GAT CAA ATG GCT CAA-3’	1165-1185	NM_024080	
Trpm8 3’ primer	5’-GGT GCT GAA GGC TTT GTA TAG-3’	1274-1294	NM_024080	
Trpm8 reporter probe	5’-Ln-ATG GCA TTG CTC ACA ATT TCA TCC CCA-3’	1237-1263	NM_024080	
Trpm8 quencher probe	5’-GTG AGC AAT GCC AT-3’	1250-1263	NM_024080	

Ln, lanthanide label (europium or terbium chelate); Q, quencher molecule.

Supplemental Table 3. PCR conditions of the real-time PCR assays.

Target mRNA	**Primers (nmol/L)**	**dNTPs (Fermentas, Vilnius, Lithuania) (mmol/L)**	**PCR polymerase (U/µL)**	**$MgCl_2$ (mmol/L)**	**Reporter probe (nmol/L)**	**Quencher probe (nmol/L)**	**Reaction volume (µL)**	**Template volume (µl)**	**Sample cDNA dilution**
BMP6	100	0.2	AmpliTaq® Gold DNA Polymerase, 0.025	2.5	4	170	25	2.5	1:10 or not diluted
FGF-8b	100	0.2	AmpliTaq® Gold DNA Polymerase, 0.025	2.5	17	170	25	2.5	not diluted
KLK2	100	0.2	HotMaster™ Taq DNA Polymerase, 0.016	included in the PCR buffer	17	170	25	2.5	1:10000
KLK3	100	0.2	HotMaster™ Taq DNA Polymerase, 0.016	included in the PCR buffer	17	170	25	2.5	1:10000
KLK4	100	0.2	AmpliTaq® Gold DNA Polymerase, 0.025	2.5	17	170	25	2.5	1:1000
KLK15	100	0.2	AmpliTaq® Gold DNA Polymerase, 0.016	2.5	17	170	25	5	not diluted
mmPSA	100	0.2	AmpliTaq® Gold DNA Polymerase, 0.025	2.5	17	170	25	2.5	not diluted
MSMB	100	0.1	AmpliTaq® Gold DNA Polymerase, 0.025	2.5	17	170	25	2.5	1:1000
PCA3	100	0.2	AmpliTaq® Gold DNA Polymerase, 0.025	2.5	17	170	25	2.5	1:100
PSCA	100	0.2	HotMaster™ Taq DNA Polymerase, 0.016	included in the PCR buffer	17	170	25	5	1:100
Trpm8	100	0.2	HotMaster™ Taq DNA Polymerase, 0.016	included in the PCR buffer	17	170	25	5	1:1000

Supplemental Table 4. Standard curve concentrations in real-time PCR assays.

	Range (molecules per mL of template)		
Target mRNA	**Lowest concentration**	**Highest concentration**	**Total number of points on standard curve**
BMP6	2.5×10^3	2.5×10^7	4
FGF-8b	0.1×10^3	1.3×10^5	3
KLK2	1.5×10^3	1×10^7	6
KLK3	2.5×10^3	2×10^{11}	8
KLK4	0.5×10^3	5×10^7	5
KLK15	1.5×10^3	1×10^7	6
MSMB	1×10^4	1×10^8	4
PCA3	1.3×10^3	2.5×10^{11}	7
PSCA	2.5×10^3	1×10^7	5
Trpm8	1.5×10^3	1×10^7	6

Concentrations are shown as per milliliter of template.

Supplemental Table 5. Association of gene expression with percentage of cancer in the tissue sample.

mRNA	Percentage of cancer <50% (n=57) (log_{10}copies/µg total RNA)	Percentage of cancer ≥50% (n=28) (log_{10}copies/µg total RNA)	Fold difference in medians	Univariate P value
BMP6	7.50 (6.79, 9.04)	7.41 (6.59, 8.22)	0.90	0.4
FGF-8b> LOD	15 (26%)	13 (46%)	NA	0.068
KLK2	8.52 (8.21, 8.94)	8.59 (8.27, 8.87)	1.27	0.8
KLK3	9.52 (8.76, 9.92)	9.12 (8.68, 9.75)	0.39	0.4
KLK4	9.15 (8.44, 9.54)	9.07 (8.30, 9.66)	0.86	0.9
KLK15	5.23 (4.72, 5.82)	5.01 (4.81, 6.07)	0.74	0.4
MSMB	9.99 (9.03, 10.5)	9.52 (8.71, 10.4)	0.34	0.3
PCA3	7.66 (7.14, 8.19)	7.99 (7.23, 9.06)	2.23	0.16
PSCA	7.27 (6.60, 8.02)	6.77 (6.30, 7.38)	0.33	0.12
Trpm8	7.87 (7.14, 8.29)	7.78 (6.98, 8.30)	0.68	0.5

Data are given as median (interquartile range) or frequency (percentage). NA, not available. In statistical analyses samples were categorized as negative or positive for *FGF-8* mRNA. For the other genes, the rank of expression level was entered as the independent variable to account for their skewed distribution.