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Published in:
Urology

DOI:
10.1016/j.urology.2013.11.005

2014

Cancer-associated changes in expression of *TMPRSS2-ERG*, *PCA3* and *SPINK1* in histologically benign tissue from cancerous versus non-cancerous prostatectomy specimens

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Word count for abstract: 248

Word count for manuscript text: 2995

Keywords: Prostatic Neoplasms, Gene Expression, Reverse Transcriptase Polymerase Chain Reaction, TMPRSS2-ERG, PCA3, SPINK1

Research support: Academy of Finland (Project 206690), European Union 6th Framework contract LSHC-CT-2004-503011 (P-Mark), National Cancer Institute (R33 CA 127768-03, R01 CA160816-02, P50-CA92629), Swedish Cancer Society (11-0624), the Sidney Kimmel Center for Prostate and Urologic Cancers, David H Koch through the Prostate Cancer Foundation, the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre based at Oxford University Hospitals NHS Trust and University of Oxford, and Fundación Federico SA.

Conflicts of interest:

Riina-Minna Väänänen: none

Hans Lilja: Dr Lilja holds patents for free PSA, intact PSA and hK2 assays.

Leni Kauko: none

Pauliina Helo: none

Henna Kekki: none

Angel M Cronin: none

Andrew J Vickers: none

Martti Nurmi: none

Kalle Alanen: none

Anders Bjartell: none

Kim Pettersson: none

ABSTRACT

Objective: To investigate whether mRNA expression of *TMPRSS2-ERG* fusion gene, a suggested prostate cancer (PCa) biomarker, was specific to cancerous lesions alone and to study the expression of *SPINK1* and *PCA3* mRNAs in the same cohort to also explore the proposed mutual exclusivity of *TMPRSS2-ERG* and *SPINK1* expression.

Methods: Levels of two *TMPRSS2-ERG* transcripts, *PCA3,* and *SPINK1* mRNAs were measured with highly standardized RT-qPCR assays in cystoprostatectomy specimens from 19 invasive bladder cancer patients and in 174 radical prostatectomy (RP) samples [88 histologically benign prostate (HBP) tissues and 86 from cancerous lesions] from 87 patients with clinically localized PCa.

Results: Expression of *TMPRSS2-ERG* transcripts was detected in 45/88 (51%) HBP tissues from RP specimens, and more frequently (57/86, 66%) found in cancerous lesions. By contrast, *TMPRSS2-ERG* expression was detected in only 2/19 (11%) cystoprostatectomy specimens, both with incidental PCa foci elsewhere in the gland. Similar trends of changes in the expression of *PCA3* and *SPINK1* were present in HBP tissue from RP compared to cystoprostatectomy specimens.

Conclusions: Although expression of *TMPRSS2-ERG*, *SPINK1*, and *PCA3* mRNA is higher or more frequently found in cancerous lesions, HBP tissues from patients with clinically localized PCa manifest molecular, mRNA level changes that are absent in cystoprostatectomy specimens lacking incidental PCa foci, or infrequent in cystoprostatectomy specimens containing incidental PCa. If this finding is replicated, these molecular assays could be used to inform men with negative biopsies about the likelihood of cancerous lesions in unsampled regions and hence the need for repeat biopsy.

INTRODUCTION

Fusion between genes *TMPRSS2* (transmembrane protease serine 2) and *ERG* (ETV related gene) has been suggested as a novel, cancer-specific marker for prostate cancer (PCa)[1]. With the high prevalence of PCa, it may be the most common cancer-associated genetic rearrangement as studies have shown it to be present in about 40-70% of PCa cases. This gene fusion has been identified in a subset of prostatic tumors with potentially distinct disease characteristics. The details of those characteristics are still debated as contradictory results on different transcript isoforms and their effects on prognosis have been reported[2,3]. The cancer specificity of this gene fusion has been questioned as *TMPRSS2-ERG* expression has been detected also in histologically benign tissue of cancerous prostates[4,5,6], but cancer-related changes of gene expression in non-neoplastic tissue of cancer-affected prostates are not unheard of and have been reported for other biomarkers as well[7,8,9].

Another subgroup of PCa was suggested when *SPINK1* mRNA, often studied in conjunction with pancreatic diseases but also overexpressed in PCa[10], was found to be overexpressed preferentially in cancers lacking *TMPRSS2-ERG* fusion[11,12]. However, a recent study by Leinonen et al contradicts this by showing no signs of their mutually exclusive expression[13].

As most of the previous *TMPRSS2-ERG* studies have been DNA- and FISH-based, we set out to study the mRNA transcript levels of the fusion gene, targeting two of the over 20 transcript isoforms that have been reported[14]: transcripts III and VI. *TMPRSS2-ERG* III is the most commonly expressed isoform[1] and *TMPRSS2-ERG* VI was chosen for its claimed association with aggressive disease[15]. For this purpose, we developed highly sensitive, truly quantitative and internally standardized reverse-transcription PCR (RT-qPCR) assays for both *TMPRSS2-ERG* transcript

isoforms as well as for *SPINK1* mRNA, employing a previously described assay concept[16]. The mRNA levels of these three markers were measured in 86 cancerous prostate tissues and 88 histologically benign prostate tissues (HBP) from 87 PCa patients, and in prostatic tissue specimens obtained by cystoprostatectomy from 19 patients with invasive cancer of the urinary bladder and with no history of PCa. In addition to measuring the absolute levels of expression with a truly quantitative method, our aim was to find out whether the previously reported[4,5,6], occasional presence of the gene fusion in benign tissue in men with PCa was evidence of a field effect or due to the gene fusion not being truly cancer specific. We also sought to explore the claimed mutual exclusivity between *SPINK1* and *TMPRSS2-ERG* fusion gene and whether there were alterations in their expression levels correlating to the neoplastic characteristics of the tissue. Association with PCa stage and tumor percentage was also assessed for all transcripts.

Our previously reported data on higher-than-expected levels of *PCA3* in HBP tissue of cancer-affected prostates compared to cancerous tissues[17] raised questions on the field effect hypothesis regarding that gene. Those issues were also explored further in this study by measuring the mRNA levels of *PCA3* in the 19 cystoprostatectomy specimens and by comparing those levels to the previously obtained values of *PCA3* expression in HBP and cancerous tissue of these 87 PCa patients.

MATERIALS AND METHODS

Tissue samples

We obtained 174 prostate tissue samples from 87 PCa patients immediately after radical prostatectomy (RP) at Turku University Hospital, Turku, Finland. Two small sample wedges were taken from each prostate, one intending to sample the suspected cancer area and the other the

control area. A small tissue sample size ensured the best possible homogeneity of material. Based on the histological examination of the immediately adjacent tissue surrounding each sampling site, an experienced senior genitourinary pathologist classified 76 samples as histologically benign tissue, 12 as prostatic intraepithelial neoplasia (PIN), and 86 as cancerous tissue ranging from Gleason grade 2 to 5. The PIN samples were considered as histologically benign tissue in all subsequent analyses unless stated otherwise. The estimated median proportion of cancerous tissue in cancerous samples was 30% (interquartile range: 10, 55). Patient characteristics are shown in Supplemental Table 1 and described previously[17]. None of the patients received prior radiation therapy. Samples were stored as previously described[18].

We also obtained prostate tissue samples from 19 patients undergoing cystoprostatectomy for treatment of invasive bladder cancer at Skåne University Hospital, Malmö, Sweden. Samples were obtained from the peripheral apical or dorsolateral portion of the prostate within 15 minutes after surgical removal and stored deep-frozen. Incidental, small foci of prostate adenocarcinomas were found upon further histopathological assessment in 12/19 of the cystoprostatectomy specimens but not anywhere close to the areas sampled for RNA isolation. Seven cystoprostatectomy specimens contained no evidence of incidental PCa in the prostate.

The study protocol was approved by local Ethics committees and it was in accordance with the Helsinki Declaration of 1975, as revised in 1996, with written informed consent obtained from each participant.

RNA extraction and reverse transcription

All tissue samples were processed as previously described[18]. RNA extraction protocol included the addition of a known amount of artificial RNA[19] to the samples to act as an internal control, tracking the inherent loss of RNA material throughout the process.

Real-time qPCR

Expression levels of *SPINK1* and *PCA3* mRNAs and *TMPRSS2-ERG* fusion transcripts types III and VI were measured with optimized RT-qPCR assays using target-specific oligonucleotide probes and time-resolved fluorometry in detection as described previously[18] and in Supplemental Tables 2, 3, and 4. *KLK3* mRNA levels were also determined with an RT-qPCR assay with the same concept[16,21,22] to ensure the presence of prostate cells. Data analysis is described in detail in Supplemental Data 1.

RESULTS

KLK3

There were no significant differences in *KLK3* mRNA levels when we compared the expression in cancerous versus HBP tissue from RP specimens (p=0.2) from men with clinically localized PCa as previously reported[17]. However, *KLK3* expression tended to be higher in HBP tissue from RP compared to cystoprostatectomy specimens (p=0.032).

TMPRSS2-ERG

Overall, there was evidence of *TMPRSS2-ERG* III expression in 55/87 (63%) and *TMPRSS2-ERG* VI expression in 51/87 (59%) men with clinically localized PCa. Individual tissue samples contained either both of the fusion transcript types, only one of them or neither. Both transcripts

were detected in 43/87 (49%) patients, with at least one of the fusion transcripts detected in 63/87 (72%) of these patients.

Of the 88 HBP tissue samples from RP specimens from men with clinically localized PCa, more than half (45/88 or 51%) showed expression of at least one of the *TMPRSS2-ERG* transcripts (Table 1). Expression of either or both of the *TMPRSS2-ERG* transcripts was detected in 57/86 (66%) cancerous tissue samples from the RP specimens. By contrast, *TMPRSS2-ERG* expression was not detected in prostatic tissue from 17 patients with invasive bladder cancer treated with cystoprostatectomy, whereas it was detectable in prostate tissue from two cystoprostatectomy specimens which contained foci of incidental PCa elsewhere in the gland.

Trends of field effect were observed particularly in the paired samples. Of the 48 men from whom both a cancerous and benign sample were obtained, 30 manifested either or both of the *TMPRSS2-ERG* transcripts in the cancerous sample and 21/30 (70%) of them had detectable *TMPRSS2-ERG* transcripts also in the matched HBP tissue. In addition, evidence of multi-clonality was implicated in glands presenting with two cancerous samples. Of the 19 men from whom two cancerous RP samples were obtained, 2 manifested only *TMPRSS2-ERG* III mRNA in one of the samples and only *TMPRSS2-ERG* VI mRNA in the other. Concordance of expression of the two *TMPRSS2-ERG* transcripts between the paired samples is described in full detail in Supplemental Tables 5 and 6.

Both *TMPRSS2-ERG* fusion transcripts were statistically significantly more frequently detected in cancerous than in HBP tissue of the RP specimens from men with clinically localized PCa (for *TMPRSS2-ERG* III, p=0.022 and for *TMPRSS2-ERG* VI, p=0.026). Also the differences in the presence of fusion transcripts between HBP tissue from prostates with clinically localized PCa and cystoprostatectomy specimens were statistically significant for both fusion transcripts (p=0.020 for

TMPRSS2-ERG III and p=0.033 for *TMPRSS2-ERG* VI). There was no statistically significant difference in *TMPRSS2-ERG* III and VI levels between PIN samples and HBP tissue from RP specimens (p=0.2 and 0.11 respectively).

Among the fusion transcript positive samples of the RP specimens from clinically localized PCa, the expression levels of each isoform varied over 5–6 orders of magnitude with no statistically significant difference of mRNA levels between the cancerous and histologically benign groups (Figure 1). The *TMPRSS2-ERG* VI expression levels of the two cystoprostatectomy samples in which the expression of this mRNA was detectable, were $3.5x10^4$ and $4.6x10^5$ mRNA copies per µg of total RNA. The latter sample also presented *TMPRSS2-ERG* III mRNA expression on the level of $3.2x10^3$ mRNA copies per µg of total RNA.

Within the samples where *TMPRSS2-ERG* VI mRNA was detectable, isoform VI levels were statistically significantly higher if the same sample also showed detectable III isoform expression (Figure 2). This phenomenon was seen both in cancerous (p=0.004 by Mann-Whitney) and HBP samples (p=0.001). However, no statistically significant difference (p=0.18) was observed in *TMPRSS2-ERG* III levels regarding whether the samples also expressed *TMPRSS2-ERG* VI or not (Figure 2).

TMPRSS2-ERG expression did not associate with Gleason grade of the tissue sample (p=0.5 for both III and VI) but advanced pathologic stage was positively associated with the presence of *TMPRSS2-ERG* III (p=0.003), and *TMPRSS2-ERG* VI mRNAs (p=0.020) (Supplemental Table 7). There was no significant association between *TMPRSS2-ERG* III and VI mRNA levels and the cancer cell percentage of the tissue samples (p=0.8 and p=0.7, respectively).

SPINK1

All samples except one cystoprostatectomy sample contained measurable *SPINK1* mRNA (Figure 3). The cancerous tissue from RP specimens had significantly higher mRNA levels (2.9-fold difference in medians, p=0.047) than the HBP tissue of RP specimens from men with clinically localized PCa. Furthermore, *SPINK1* mRNA levels were statistically significantly lower in the cystoprostatectomy samples than in the HBP samples from RP specimens (3.4-fold difference in medians, p=0.010 after adjusting for clustering). The difference in median expression between cancerous tissue of RP specimens and cystoprostatectomy samples was 10-fold (p<0.001).

Neither Gleason grade of the tissue sample (p=0.4) nor advanced pathologic stage (p=0.3) was significantly associated with *SPINK1* mRNA expression levels (Supplemental Table 7). There was no significant association between *SPINK1* mRNA levels and the cancer cell percentage of the tissue samples (p=0.3).

The presence of *TMPRSS2-ERG* transcripts did not correlate with *SPINK1* levels (p=0.8 for *TMPRSS2-ERG* III and p=0.6 for *TMPRSS2-ERG* VI), but there was a statistically significant association between *SPINK1* mRNA levels and *TMPRSS2-ERG* III and VI levels (p=0.001 and p=0.041, respectively) in those samples where *TMPRSS2-ERG* fusion transcripts were detected.

PCA3

PCA3 mRNA was detectable in all 19 cystoprostatectomy samples. The median *PCA3* mRNA level in those samples was 7.9×10^4 mRNA copies per µg of total RNA, which is 613-fold lower than the previously reported median *PCA3* expression level in cancerous tissues[17] and 107-fold lower than the previously reported median[17] in HBP tissue of cancer-affected prostates. These differences were statistically significant (p<0.0001), but there was no statistically significant difference between

PCA3 mRNA levels in cystoprostatectomy samples from cancer-free prostates (n=7) and levels in samples from prostates with incidental cancer foci elsewhere in the prostate (n=12) (p=0.18).

DISCUSSION

Although a significantly higher frequency of *TMPRSS2-ERG* transcripts was found in the cancerous tissue compared to the HBP tissue from the prostates harboring clinically localized cancer, the frequency of *TMPRSS2-ERG* expression remained high in samples from histologically benign areas. In contrast, fusion gene transcripts were detectable only rarely in cystoprostatectomy samples from bladder cancer patients and never without concomitant incidental tumor foci present in the same prostate. Thus *TMPRSS2-ERG* expression can be detected in histologically normal prostate tissue obtained from a prostate in which tumors were detected. A similar trend was seen in *PCA3* and *SPINK1* mRNA levels.

The presence of *TMPRSS2-ERG* fusions has been widely considered to be a cancer-specific event[1], but we detected *TMPRSS2-ERG* III and/or VI mRNAs also in 51% of HBP tissues of RP specimens from prostates with clinically localized PCa (compared to 66% in the cancerous RP samples). The phenomenon of *TMPRSS2-ERG* positive, benign-appearing prostate tissue has been reported before on a few occasions[4,5,6]. In previous studies, it has mainly been considered an anomaly, but a conceivable explanation could be a carcinogenic field effect[23], which suggests that a larger area than just the tumor focus is originally changed in terms of neoplastic events due to a carcinogenic signal that has affected the entire area[24]. This type of area can appear histologically normal, albeit molecular changes may still be detectable. Field effect-related observations in PCa[8,9,25,26] led us to the hypothesis that the HBP tissue of PCa patients in this study may have undergone molecular changes with regard to *PCA3* and *SPINK1* genes and *TMPRSS2-ERG* fusion events even though the

histological examination defined the samples as non-neoplastic. This is further supported not only by the fact that the two cystoprostatectomy samples that presented *TMPRSS2-ERG* expression, were taken from prostates that in pathologic sectioning studies were found to contain incidental prostate tumor foci, but also by the higher likelihood of the HBP samples to contain detectable *TMPRSS2-ERG* mRNA if the matched cancerous sample was also *TMPRSS2-ERG* fusion-positive.

The previously reported ranges of 10-100-fold overexpression of *PCA3* in cancerous tissue[27,28] did not seem to be in line with our recent results where the control tissue was HBP tissue from prostates with clinical PCa and only a 5.7-fold overexpression was observed[17]. However, in light of this study where we determined the *PCA3* expression in benign prostate tissue from cystoprostatectomy specimens and compared it with the previously measured levels of *PCA3* mRNA in tissue of cancer-affected prostates, the overexpression of *PCA3* is more pronounced when the cancerous tissue is, in fact, compared to tissue from prostates without clinical PCa. The cohort of prostates without clinical PCa is admittedly limited in this study, but nevertheless, this suggests that the *PCA3* levels may also be elevated in histologically benign areas of the cancerous prostate when compared to cancer-free prostates – as was seen here as a 107-fold difference in mRNA levels.

SPINK1 has been claimed to be overexpressed in PCa[9,29] and also to exclusively identify a subgroup of PCas lacking *TMPRSS2-ERG* fusion, although contradictory results have been reported as well[13]. In this study, we did not find them to be mutually exclusive, but did observe a positive correlation between *SPINK1* and *TMPRSS2-ERG* mRNA levels in the samples where *TMPRSS2-ERG* fusion transcripts were detected. However, measuring transcripts in tissue extracts does not allow a specific detection of different transcripts in the individual tumor cells which is a limitation with our and previous studies. A possible co-expression of gene fusions and *SPINK1* mRNA in the same tumor cells still needs to be clarified.

Despite the small size of the studied cohort, these findings reported here suggest that detection of *TMPRSS2-ERG* fusion transcripts is indicative of presence of prostatic tumor in the corresponding prostate, whether or not the sampled cells appear neoplastic in histological examinations. As it was possible to examine histologically only the immediately adjacent tissue, and not the actual tissue used for the RT-qPCR analyses, we cannot exclude the possibility that cancer cells may have resided in the tissue deemed benign using established state-of-art histopathological analyses. It however seems unlikely that this would be the case for such a large number of samples used in our study. However, this study does not reveal whether the finding of *TMPRSS2-ERG* transcripts can be made even before the emergence of the tumor. If the expression of fusion transcripts is considered an early event, it may, as an inducing carcinogenic signal, be present also before the formation of histologically evident tumor foci. This could therefore give very early information not only about the likelihood of already established disease but also about increased future risk of tumor formation in the prostate even in cases where no tumor tissue was found by the biopsy procedure. Using RT-qPCR assays for determining the molecular composition of biopsy material could conceivably constitute an informative adjunct to the traditional histological examination, and this creates an interesting option calling for future studies. Extraction of nucleic acids from prostate biopsy material is technologically feasible[30] although the amount of tissue used to extract nucleic acids would compete with the amount used for histopathological evaluation.

CONCLUSIONS

To conclude, histologically benign tissue from a prostate affected by PCa can present molecular level changes in mRNA expression regarding the fusion gene *TMPRSS2-ERG* and *PCA3* and *SPINK1* genes. Thus, molecular tools such as the markers studied here could provide a means to

identify an enhanced risk of PCa or presence of already established PCa missed by the biopsy procedure.

REFERENCES

1. Tomlins SA, Rhodes DR, Perner S, et al: Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 2005;310:644-8.

2. Demichelis F, Fall K, Perner S, et al: TMPRSS2:ERG gene fusion associated with lethal prostate cancer in a watchful waiting cohort. Oncogene 2007;26:4596–9.

3. Saramäki OR, Harjula AE, Martikainen PM, et al: TMPRSS2:ERG fusion identifies a subgroup of prostate cancers with a favorable prognosis. Clin Cancer Res 2008;14:3395–400.

4. Clark J, Merson S, Jhavar S, et al: Diversity of TMPRSS2-ERG fusion transcripts in the human prostate. Oncogene 2007;26:2667–73.

5. Furusato B, Gao CL, Ravindranath L, et al: Mapping of TMPRSS2-ERG fusions in the context of multi-focal prostate cancer. Mod Pathol 2008;21:67–75.

6. Watson SK, Woolcock BW, Fee JN, et al: Minimum altered regions in early prostate cancer progression identified by high resolution whole genome tiling path BAC array comparative hybridization. Prostate 2009;69:961-75.

7. Luo JH: Gene expression alterations in human prostate cancer. Drugs Today (Barc) 2002;38:713-9.

8. Yu YP, Landsittel D, Jing L, et al: Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. J Clin Oncol 2004;22:2790-9.

9. Kosari F, Cheville JC, Ida CM, et al: Shared gene expression alterations in prostate cancer and histologically benign prostate from patients with prostate cancer. Am J Pathol 2012;181:34-42.

10. Paju A, Hotakainen K, Cao Y, et al: Increased expression of tumor-associated trypsin inhibitor, TATI, in prostate cancer and in androgen-independent 22Rv1 cells. Eur Urol 2007;52:1670–9.

11. Tomlins SA, Rhodes DR, Yu J, et al: The role of SPINK1 in ETS rearrangement-negative prostate cancers. Cancer Cell 2008;13:519-28.

12. Jhavar S, Brewer D, Edwards S, et al: Integration of ERG gene mapping and gene-expression profiling identifies distinct categories of human prostate cancer. BJU Int 2009;103:1256-69.

13. Leinonen KA, Tolonen TT, Bracken H, et al: Association of SPINK1 Expression and TMPRSS2:ERG Fusion with Prognosis in Endocrine-Treated Prostate Cancer. Clin Cancer Res 2010;16:2845–51.

14. Tomlins SA, Bjartell A, Chinnaiyan AM, et al: ETS gene fusions in prostate cancer: from discovery to daily clinical practice. Eur Urol 2009;56:275–86.

15. Wang J, Cai Y, Ren C, et al: Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. Cancer Res 2006;66:8347–51.

16. Nurmi J, Wikman T, Karp M, et al: High-performance real-time quantitative RT-PCR using lanthanide probes and a dual-temperature hybridization assay. Anal Chem 2002;74:3525–32.

17. Väänänen RM, Lilja H, Cronin A, et al: Association of transcript levels of 10 established or candidate-biomarker gene targets with cancerous versus non-cancerous prostate tissue from radical prostatectomy specimens. Clin Biochem 2013;46:670–4.

18. Väänänen RM, Rissanen M, Kauko O, et al: Quantitative real-time RT-PCR assay for PCA3. Clin Biochem 2008;41:103-8.

19. Nurmi J, Lilja H, Ylikoski A. Time-resolved fluorometry in end-point and real-time PCR quantification of nucleic acids. Luminescence 2000;15:381–8.

20. Cerveira N, Ribeiro FR, Peixoto A, et al: TMPRSS2-ERG gene fusion causing ERG overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. Neoplasia 2006;8:826–32.

21. Rissanen M, Helo P, Väänänen RM, et al: Novel homogenous time-resolved fluorometric RT-PCR assays for quantification of PSA and hK2 mRNAs in blood. Clin Biochem 2007;40:111-8.

22. Nurmi J, Ylikoski A, Soukka T, et al: A new label technology for the detection of specific polymerase chain reaction products in a closed tube. Nucleic Acids Res 2000;28:E28.

23. Nonn L, Ananthanarayanan V, Gann PH. Evidence for field cancerization of the prostate. Prostate 2009;69:1470–9.

24. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer 1953;6:963–8.

25. Mazzucchelli R, Barbisan F, Santinelli A, et al: Immunohistochemical expression of prostate tumor overexpressed 1 in cystoprostatectomies with incidental and insignificant prostate cancer. Further evidence for field effect in prostatic carcinogenesis. Hum Pathol 2011;42:1931-6.

26. Popa I, Fradet Y, Beaudry G, et al: Identification of PCA3 (DD3) in prostatic carcinoma by in situ hybridization. Mod Pathol 2007;20:1121-7.

27. Bussemakers MJ, van Bokhoven A, Verhaegh GW, et al: DD3: a new prostate-specific gene, highly overexpressed in prostate cancer. Cancer Res 1999;59:5975–9.

28. Hessels D, Klein Gunnewiek JM, van Oort I, et al: DD3(PCA3)-based molecular urine analysis for the diagnosis of prostate cancer. Eur Urol 2003;44:8–15.

29. Laxman B, Morris DS, Yu J, et al: A first-generation multiplex biomarker analysis of urine for the early detection of prostate cancer. Cancer Res 2008;68:645–9.

30. Fukabori Y, Yoshida K, Nakano K, et al: Preparation of a single prostate needle biopsy specimen for histological diagnosis and RNA analysis. J Urol 2006;176:1204–7.

Table 1. Frequencies of *TMPRSS2-ERG* III and VI mRNA expression detected in histologically benign tissues of prostates with clinical PCa, PIN tissue, cancerous prostate tissues and in tissues of clinical PCa-free prostates. PIN, prostatic intraepithelial neoplasia; RP, radical prostatectomy.

	Number of samples				
	Only *TMPRSS2-ERG* III mRNA was detected	Only *TMPRSS2-ERG* VI mRNA was detected	Both *TMPRSS2-ERG* III and VI mRNAs were detected	Neither *TMPRSS2-ERG* III nor VI mRNA were detected	Total
Histologically benign tissue from RP specimens (including the 12 PIN samples)	11 (13%)	10 (11%)	24 (27%)	43 (49%)	88
PIN tissue from RP specimens	0 (0%)	4 (33%)	3 (25%)	5 (42%)	12
Cancerous prostate tissue from RP specimens	10 (12%)	8 (9%)	39 (45%)	29 (34%)	86
Prostate tissue from cystoprostatectomy specimens	0 (0%)	1 (5%)	1 (5%)	17 (89%)	19

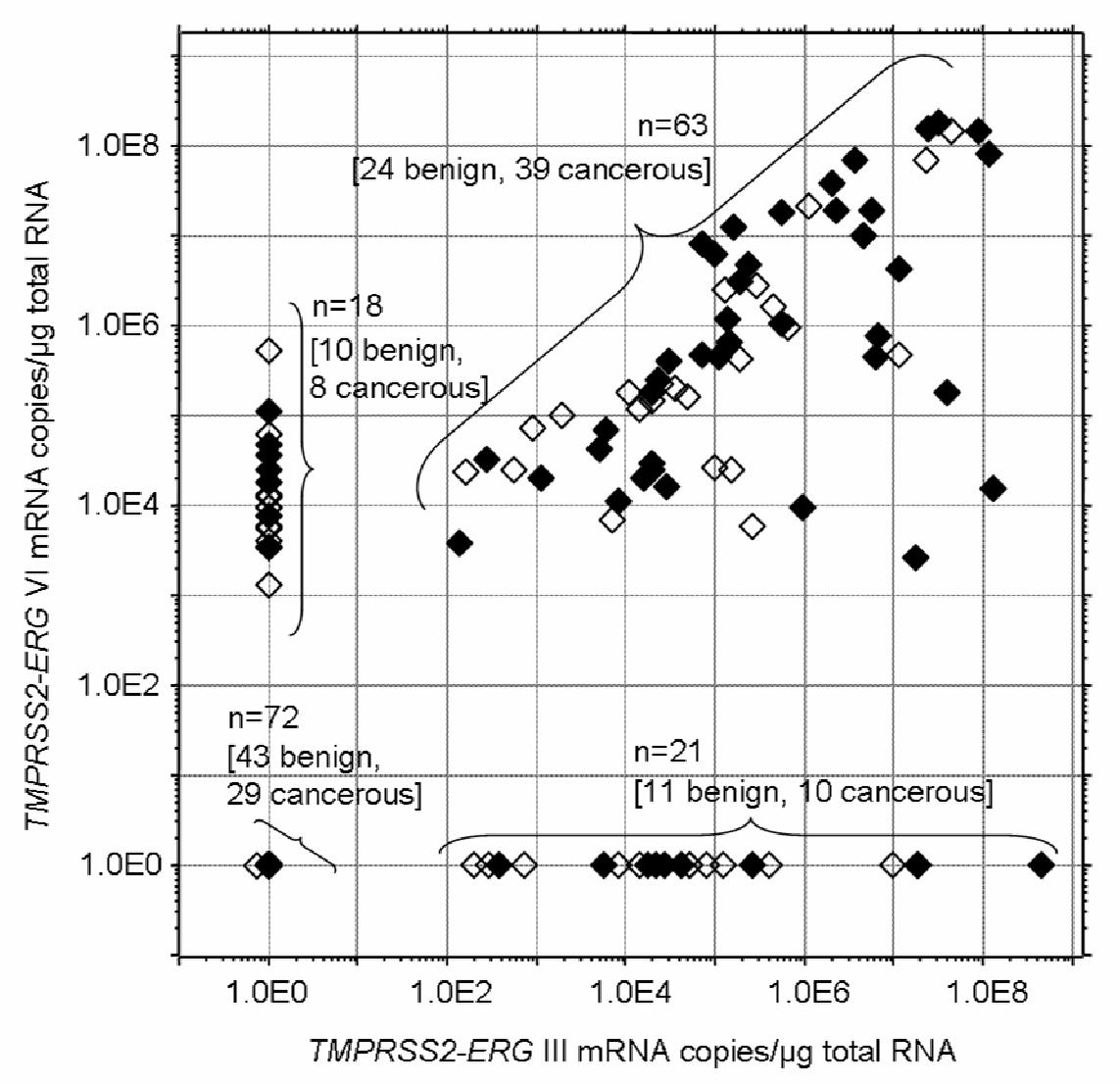

Figure 1.

TMPRSS2-ERG III and VI mRNA levels in 174 radical prostatectomy samples. The open diamonds denote histologically benign tissue samples and the closed diamonds represent cancerous sample. When mRNA was not detected, the sample was given the value 1.

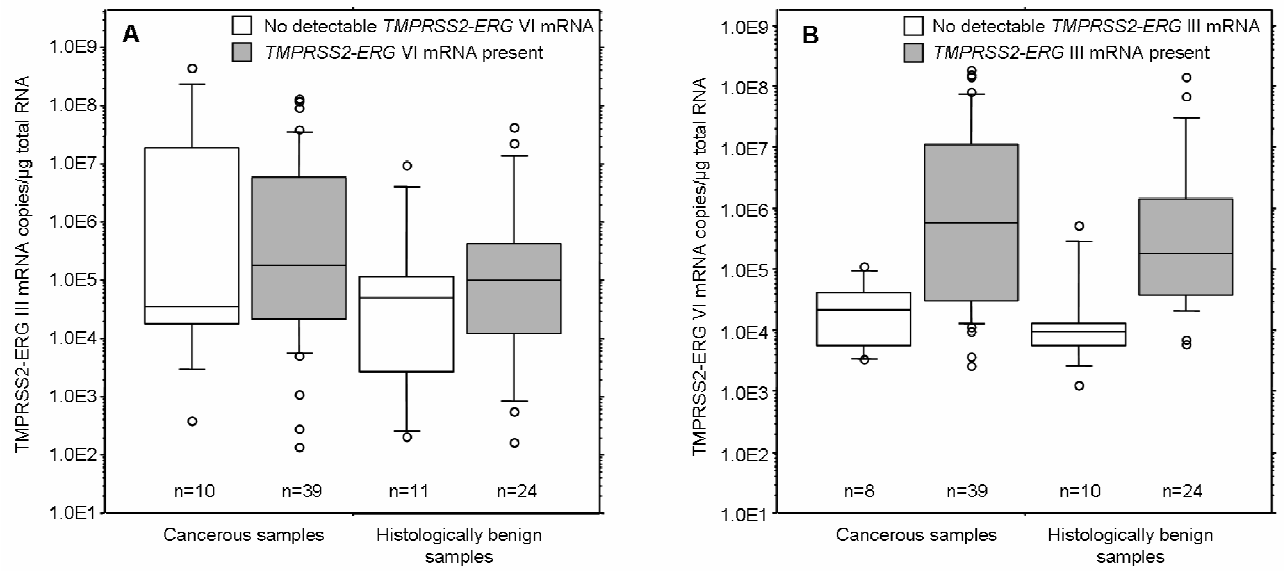

Figure 2.

A. *TMPRSS2-ERG* III mRNA levels in cancerous and histologically benign samples with (grey) or without (white) detectable *TMPRSS2-ERG* VI mRNA expression.

B. *TMPRSS2-ERG* VI mRNA levels in cancerous and histologically benign samples with (grey) or without (white) detectable *TMPRSS2-ERG* III mRNA expression.

The 10/25/50/75/90th percentiles are marked in the figures and open circles denote the outlier values.

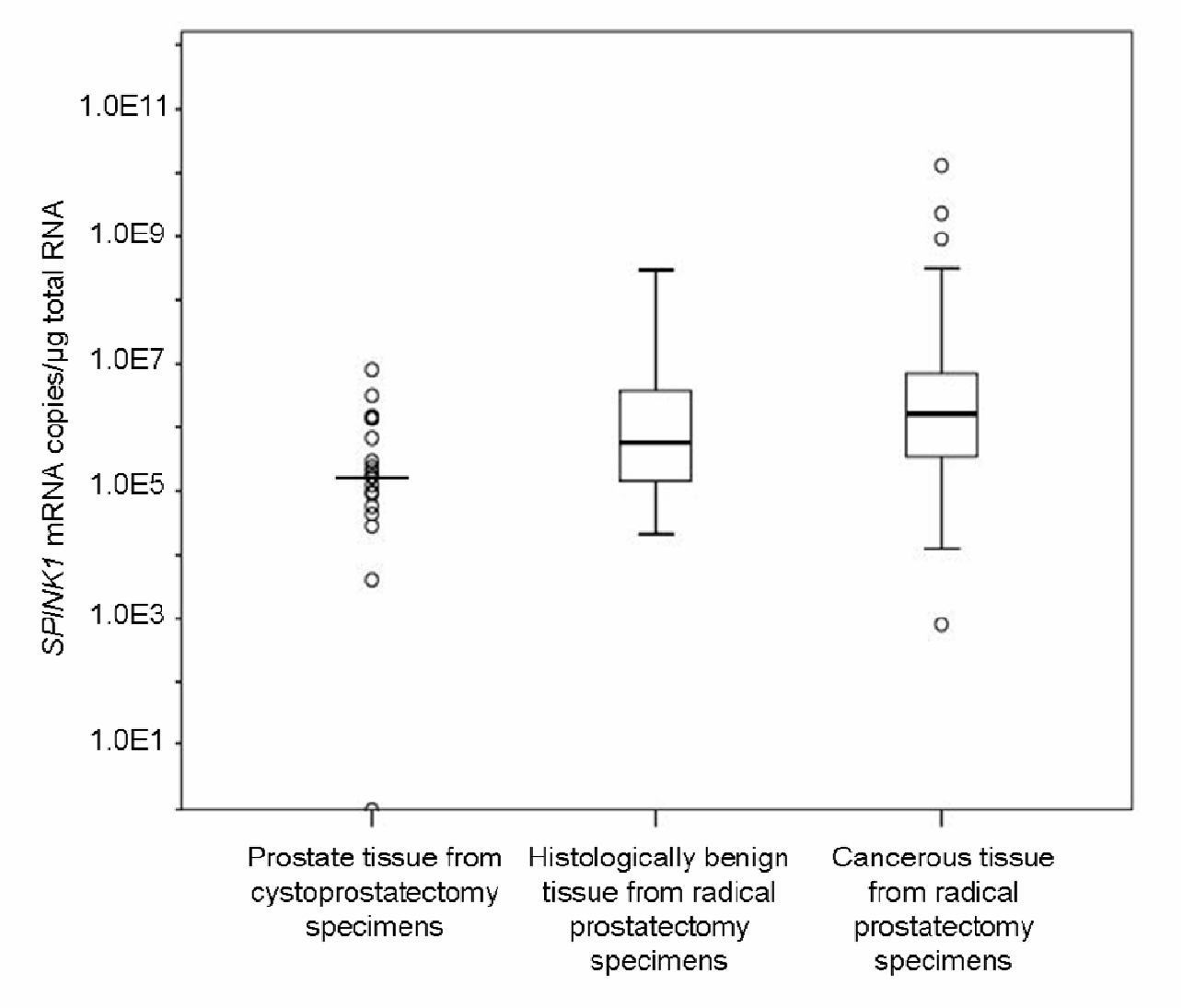

Figure 3.

SPINK1 mRNA levels in cystoprostatectomy specimens and histologically benign or cancerous areas from radical prostatectomy specimens. For the cystoprostatectomy group, the horizontal line denotes the median value and the open circles denote *SPINK1* mRNA values in individual samples. For the other two groups the 10/25/50/75/90th percentiles are marked in the figure and open circles denote the outlier values.

Supplemental Data 1. Data analysis.

Samples were only considered positive if all three PCR replicates gave higher values than the limit of detection (LOD) of the assay. The ΔC_ts were calculated for target-internal control differences and standard plots were formed accordingly [17].

Statistical analyses were performed with Stata 12.0 (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 histology and presence (detectable expression) or absence (expression below the LOD) of *TMPRSS2-ERG* III and VI mRNA, we used univariate logistic regression with the cluster option to account for multiple samples per patient. For absolute levels of *TMPRSS2-ERG* III and VI and for *SPINK1* and *PCA3*, we used linear regression after log transformation, again using clustering to account for the use of multiple samples.

Supplemental Table 1. Patient characteristics for 87 men with clinically localized PCa.

	Number of patients (percentage)
Pathologic stage[1]	
pT2	36/87 (45%)
pT3 or pT4	44/87 (55%)
Pathologic Gleason score[2]	
≤ 6	46/87 (57%)
7	22/87 (27%)
≥ 8	13/87 (16%)
Type of tissue samples the patient contributed	
2 histologically benign	20/87 (23%)
1 histologically benign, 1 cancerous	48/87 (55%)
2 cancerous	19/87 (22%)

[1]Pathologic stage was unknown for 7 patients
[2]Pathologic Gleason score was unknown for 6 patients

Supplemental Table 2. Oligonucleotides used in real-time PCR assays in this study.

Oligonucleotide	Sequence	Location	Database sequence
KLK3 5' primer	5'-TGAACCAGAGGAGTTCTTGAC-3'	523-543	X05332
KLK3 3' primer	5'-CCCAGAATCACCCGAGCAG-3'	667-685	X05332
KLK3 reporter probe	5'-Ln[1]-CCTTCTGAGGGTGAACTTGCGC-3'	596-617	X05332
KLK3 quencher probe	5'-AATCACCCTCAGAAGG-Q[2]-3'	600-601, 604-617	X05332
mmPSA 5' primer	5'-TGAACCAGAGGAGTTCTTGCA-*3'*	523-543	X05332[3]
mmPSA 3' primer	5'-CCCAGAATCACCCGAGCGA-*3'*	667-685	X05332[3]
mmPSA reporter probe	5'-Ln[1]-CCTTCTGAGGGTGATTGCGCAC-3'	594-601, 604-617	X05332[3]
mmPSA quencher probe	5'-AATCACCCTCAGAAGG-Q[2]-3'	600-601, 604-617	X05332[3]
PCA3 5' primer	5'-GGTGGGAAGGACCTGATGATAC-3'	95-116	AF103907
PCA3 3' primer	5'-GGGCGAGGCTCATCGAT-3'	505-521	AF103907
PCA3 reporter probe	5'-Ln[1]-AGAAATGCCCGGCCGCCATC-3'	478-497	AF103907
PCA3 quencher probe	5'-CCGGGCATTTCT-Q[2]-3'	478-489	AF103907
SPINK1 5' primer	5'-GACCTCTGGACGCAGAAC-3'	96-113	NM_003122
SPINK1 3' primer	5'-GTAACA TTTGGCCTCTCTTCC-3'	199-219	NM_003122
SPINK1 reporter probe	5'-Ln[1]-AAGGTAACAGGCATCTTTCTTCTCAGTG-3'	124-151	NM_003122
SPINK1 quencher probe	5'-TGCCTGTTACCTT-Q[2]-3'	124-136	NM_003122
TMPRSS2-ERG III 5' primer	5'-TAGGCGCGAGCTAAGCAGGAG-3'	4-24	NM_005656.3
TMPRSS2-ERG III 3' primer	5'-GTAGGCACACTCAAACAACGACTGG-3'	338-362	NM_004449.4
TMPRSS2-ERG III reporter probe	5'-AGCGCGGCAGGAAGCCTTATCAGTT-3'	57-64; 310-326	NM_005656.3; NM_004449.4
TMPRSS2-ERG III quencher probe	5'-TTCCTGCCGCGCT-Q[2]-3'	57-64; 310-314	NM_005656.3; NM_004449.4
TMPRSS2-ERG VI 5' primer	5'-CGGCAGGTCATATTGAACATTCC-3'	73-95	NM_005656.3
TMPRSS2-ERG VI 3' primer	5'-GCACACTCAAACAACGACTGG-3'	338-358	NM_004449.4
TMPRSS2-ERG VI reporter probe	5'-Ln[1]-CTTTGAACTCAGAAGCCTTATCAGTTGTGA-3'	139-149; 312-330	NM_005656.3; NM_004449.4
TMPRSS2-ERG VI quencher probe	5'-GGCTTCTGAGTTCAAAG-Q[2]-3'	139-149; 312-317	NM_005656.3; NM_004449.4

[1] Ln, lanthanide label (europium or terbium chelate)

[2] Q, quencher molecule
[3] Sequence for mmPSA is KLK3 sequence X05332 with mutations as described previously by Nurmi et al[16,19,22]

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

Target mRNA	Primers (Thermo, Germany) (nmol/L)	dNTPs (Fermentas, 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
KLK3	100	0.2	HotMaster™ Taq DNA Polymerase (Eppendorf, Germany), 0.016	included in the PCR buffer	17	170	25	2.5	1:10000
mmPSA	100	0.2	AmpliTaq® Gold DNA Polymerase (Applied Biosystems, USA), 0.025	2.5	17	170	25	2.5	not diluted
SPINK1	100	0.1	AmpliTaq® Gold DNA Polymerase, 0.025	2.5	17	170	25	2.5	1:10
TMPRSS2-ERG III	100	0.2	AmpliTaq® Gold DNA Polymerase, 0.025	1.5	170	1700	10	2.5	1:10
TMPRSS2-ERG VI	500	0.4	AmpliTaq® Gold DNA Polymerase, 0.050	2.5	43	170	25	2.5	1:10 or not diluted

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
KLK3	2.5×10^3	2×10^{11}	8
SPINK1	1×10^4	1×10^8	4
TMPRSS2-ERG III	5×10^3	5×10^7	4
TMPRSS2-ERG VI	2×10^4	2×10^8	4

Supplemental Table 5.

Concordance of *TMPRSS2-ERG* expression in radical prostatectomy specimens from 48 prostate cancer cases in which there was one cancerous tissue sample matched with one sample containing only histologically benign tissue.

	n(patients)
Concordant *TMPRSS2-ERG* expression	33/48 (69%)
transcripts detected in both cancerous and HBP[1] sample	21
no transcripts detected in either cancerous or HBP[1] sample	12
Discordant *TMPRSS2-ERG* expression	15/48 (31%)
transcripts detected only in cancerous sample	9
transcripts detected only in HBP[1] sample	6
Concordant *TMPRSS2-ERG* III expression	33/48 (69%)
transcripts detected in both cancerous and HBP[1] sample	15
no transcripts detected in either cancerous or HBP[1] sample	18
Discordant *TMPRSS2-ERG* III expression	15/48 (31%)
transcripts detected only in cancerous sample	10
transcripts detected only in HBP[1] sample	5
Concordant *TMPRSS2-ERG* VI expression	37/48 (77%)
transcripts detected in both cancerous and HBP[1] sample	18
no transcripts detected in either cancerous or HBP[1] sample	19
Discordant *TMPRSS2-ERG* VI expression	11/48 (23%)
transcripts detected only in cancerous sample	7
transcripts detected only in HBP[1] sample	4
Cases where one sample manifested only *TMPRSS2-ERG* III expression and the other only *TMPRSS2-ERG* VI expression	0/48 (0%)

[1]HBP, histologically benign prostate

Supplemental Table 6.

Concordance of *TMPRSS2-ERG* expression in radical prostatectomy specimens from 19 prostate cancer cases in which two cancerous samples were matched.

	n(patients)
Concordant *TMPRSS2-ERG* expression	16/19 (84%)
transcripts detected in both samples	13
no transcripts detected in either sample	3
Discordant *TMPRSS2-ERG* expression	3/19 (16%)
Concordant *TMPRSS2-ERG* III expression	13/19 (68%)
transcripts detected in both samples	9
no transcripts detected in either sample	4
Discordant *TMPRSS2-ERG* III expression	6/19 (32%)
Concordant *TMPRSS2-ERG* VI expression	15/19 (79%)
transcripts detected in both samples	9
no transcripts detected in either sample	6
Discordant *TMPRSS2-ERG* VI expression	4/19 (21%)
Cases where one sample manifested only *TMPRSS2-ERG* III expression and the other only *TMPRSS2-ERG* VI expression	2/19

Supplemental Table 7. Association of gene expression with pathologic stage of the prostate sample.

Target gene	pT2 (n=72) (log_{10}copies/µg total RNA)	pT3/4 (n=88) (log_{10}copies/µg total RNA)	P value
SPINK1	13.4 (11.8, 15.2)	14.1 (12.5, 15.6)	0.3
TMPRSS2-ERG III > LOD[1]	25 (35%)	55 (63%)	0.003
TMPRSS2-ERG VI > LOD[1]	26 (36%)	52 (59%)	0.020

Data are given as median (interquartile range) or frequency (percentage).

[1]LOD, limit of detection.