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Research Article

Loss of PTEN Is Associated with Aggressive Behavior in ERG-Positive Prostate Cancer

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Abstract

Background: The associations of ERG overexpression with clinical behavior and molecular pathways of prostate cancer are incompletely known. We assessed the association of ERG expression with AR, PTEN, SPINK1, Ki-67, and EZH2 expression levels, deletion, and mutations of chromosomal region 3p14 and *TP53*, and clinicopathologic variables.

Methods: The material consisted of 326 prostatectomies, 166 needle biopsies from men treated primarily with endocrine therapy, 177 transurethral resections of castration-resistant prostate cancers (CRPC), and 114 CRPC metastases obtained from 32 men. Immunohistochemistry, FISH, and sequencing was used for the measurements.

Results: ERG expression was found in about 45% of all patient cohorts. In a multivariate analysis, ERG expression showed independent value of favorable prognosis ($P = 0.019$). ERG positivity was significantly associated with loss of PTEN expression in prostatectomy ($P = 0.0348$), and locally recurrent CRPCs ($P = 0.0042$). Loss of PTEN expression was associated ($P = 0.0085$) with shorter progression-free survival in ERG-positive, but not in negative cases. When metastases in each subject were compared, consistent ERG, PTEN, and AR expression as well as *TP53* mutations were found in a majority of subjects.

Conclusions: A similar frequency of ERG positivity from early to late stage of the disease suggests lack of selection of ERG expression during disease progression. The prognostic significance of PTEN loss solely in ERG-positive cases indicates interaction of these pathways. The finding of consistent genetic alterations in different metastases suggests that the major genetic alterations take place in the primary tumor.

Impact: Interaction of PTEN and ERG pathways warrants further studies. *Cancer Epidemiol Biomarkers Prev*; 22(12); 2333–44. ©2013 AACR.

Introduction

Gene fusions involving *TMPRSS2* (transmembrane protease, serine 2) and ETS family transcription factors are common in prostate cancer. The most common fusion occurs between *TMPRSS2* and *ERG* [v-ets erythroblastosis

virus E26 oncogene homolog (avian)], in approximately 50% of clinical prostate cancers (1). *TMPRSS2* is expressed in normal and neoplastic prostate tissue, but its exact biologic activity and function are unclear. The androgen response elements, located in the *TMPRSS2* promoter region, make *TMPRSS2* androgen inducible (2, 3). Fusion with *TMPRSS2* make ETS transcription factors to become androgen inducible leading to their overexpression in prostate cancer. Approximately 90% of prostate cancers with ERG overexpression have *TMPRSS2:ERG* fusion (4). Two other known gene fusions causing ERG overexpression are *SLC45A3:ERG* (solute carrier family 45, member 3) and *NDRG1:ERG* (N-myc downstream regulated 1), whose frequencies are less than 5% (5).

Discordant data have been published concerning the effects of *TMPRSS2:ERG* fusion on prognosis and survival. In prostatectomy-treated patients, some have found association with better prognosis and survival (6, 7), some with more aggressive disease (8–10), and others have not found any association (11, 12). In a recent meta-analysis of 5,074 men treated with radical prostatectomy, no association with good or bad prognosis was found (13). In

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hormonally treated patients, *TMPRSS2:ERG* fusion has not been found to predict the response to therapy, suggesting that the *TMPRSS2:ERG* fusion does not implicate hormone dependence of the cancer (14, 15).

In vitro studies have shown that activation of the androgen receptor (AR) increases ERG expression in fusion positive cells (16). It has also been suggested that ERG could disrupt androgen signaling directly by inhibiting AR expression and suppressing AR downstream target genes (17). Previous studies have suggested that *TMPRSS2:ERG* fusion is associated with deletions of *TP53* (tumor protein p53), *PTEN*, and a region in chromosome 3p14 (GRCh37/hg19, chr3 ~ 71,000,000–73,000,000 bp, UCSC Genome Browser; refs. 18, 19). The expression levels of EZH2 [enhancer of zeste homolog 2 (*Drosophila*)] and ERG have been found to be positively correlated in clinical prostate cancers. ERG can bind to the EZH2 promoter and induce its expression (17). In prostatectomy samples, SPINK1 (serine peptidase inhibitor, Kazal type 1) has been found to be almost exclusively expressed in ETS-fusion negative tumors (20). Association between AR and PTEN has also been suggested, but the results have been controversial showing both positive and negative correlations (21–24).

The aim of the study was to systematically interrogate whether ERG expression is associated with the expression of PTEN, AR, SPINK1, Ki-67, and EZH2, and with the genomic copy number and mutations of *TP53* and chromosome region 3p14 in large cohorts (totaling over 800 samples and 701 patients) of prostate cancers of different stages. A separate goal was to study clonality of prostate cancer metastases.

Materials and Methods

Clinical tumor samples

The use of clinical material was approved by the ethical committee of the Tampere University Hospital (TAUH, Tampere, Finland) and the National Authority for Medicolegal Affairs and the Johns Hopkins Medicine Institutional Review Board (autopsy samples).

Prostatectomy specimens. Three-hundred and twenty six formalin-fixed, paraffin-embedded (FFPE) prostate cancer samples from consecutive prostatectomies were obtained from TAUH. The clinicopathologic description of the cohort is given in Supplementary Table S1. Progression was defined as prostate-specific antigen (PSA) value 0.5 ng/mL or more in two consecutive measurements or the emergence of metastases. Fifty-one percent of the patients experienced progression.

Prostate needle biopsy specimens. One-hundred and sixty six FFPE samples from initial diagnostic prostate needle biopsies were obtained from TAUH. The material has been previously described in details (14) and in Supplementary Table S1.

Locally recurrent CRPC specimens. One-hundred and seventy seven FFPE samples of locally recurrent castration-resistant prostate cancer (CRPC) from trans-

urethral resection of the prostate (TURP) were obtained from the TAUH (Supplementary Table S1).

CRPC metastases. 114 metastases were obtained from 32 men who died of CRPC and underwent autopsy as part of the project to Eliminate Lethal Prostate Cancer (PELICAN) rapid autopsy program at the Johns Hopkins Autopsy Study of Lethal Prostate Cancer (Supplementary Tables S2 and S3). During the course of their treatment for metastatic prostate cancer all subjects received androgen deprivation therapy, either with LHRH analogue or orchiectomy. Many of them also received antiandrogens, one or more, intermittently during the course of their disease.

Representative regions of FFPE tissue blocks were chosen for tissue microarray constructed as described previously (6).

FISH

Dual- and three-color FISHs were carried out for detecting *TMPRSS2:ERG* rearrangement, as well as deletions of *TP53* and chromosomal region 3p14. Locus-specific bacterial artificial chromosome probes were used: RP11-164E1 (*ERG*), RP11-814F13 (upstream of *TMPRSS2*), RP11-367P1 (region between *TMPRSS2* and *ERG*), RP11-1057H11 (*PDZRN3*, chromosome 3p14), RP11-643D1 (*FOXPI*, chromosome 3p14), RP11-37G14 (*SHQ1*, chromosome 3p14), RP11-584A6 (*MITF*, chromosome 3p14), CTD-305405 (*TP53*). A chromosome 17 centromere-specific probe (p17H8) was used as a control in FISH analysis of *TP53* deletion. FISH was performed as previously described by Saramäki and colleagues (6). FISH signals were scored from nonoverlapping nuclei with an Olympus BX50 epifluorescence microscope equipped with a charge-coupled device camera. Stacks of nine images were captured through each filter set with the Image-Pro Plus 6.1 software (Media Cybernetics Inc) and combined to produce an RGB image with an extended depth of focus.

Immunohistochemistry

Antibodies against ERG (EPR3864; Epitomics, Inc.), SPINK1 (6E8; ref. 25), AR (318; Novocastra Laboratories Ltd.), PTEN (138G6; Cell Signaling Technology), Ki-67 (MM1; Leica Biosystems Newcastle Ltd.), and EZH2 (NCL-L-EZH2; Novocastra) were used with Power Vision+ Poly-HRP IHC kit (ImmunoVision Technologies Co.) according to the manufacturer's instructions. The protocol has previously been described (14). Slides were scanned with an Aperio ScanScope XT scanner (Aperio Technologies, Inc.), and scoring was done in a blinded fashion with the use of a virtual microscope (26). ERG, SPINK1, AR, and PTEN staining intensity was classified on a scale from 0 to 3. The immunohistochemical staining criteria are listed in Supplementary Table S4. Immunoratio (27, 28) web application was used for scoring Ki-67 and EZH2 staining.

Sequencing of TP53

TP53 exons 4 to 9 and introns 5, 7, and 8 were sequenced. Primers used are listed in Supplementary Table S5. First,

PCR was carried out with either nonmicrodissected or laser capture microdissected (LCM) samples. Cycle sequencing reaction was carried out with BigDye Terminator Cycle Sequencing kit (Applied Biosystems), and the amplified products were sequenced using ABI Prism 310 or ABI Prism 3700-based capillary gel electrophoresis. Suspected mutations were confirmed in a separately isolated and amplified LCM sample.

The mutations found by sequencing were confirmed by single-strand conformation polymorphism (SSCP). Primers used are listed in Supplementary Table S6. Purified PCR products were denatured and loaded onto 20% TBE polyacrylamide precast gels (Invitrogen) using ThermoFlow Electrophoresis Temperature Control System (Invitrogen). The gel was stained with SybrGold (Molecular Probes) and visualized using the Fluorimager SI (Vistra DNA Systems). Restriction enzyme-based sequence analyses were performed for putative mutation-containing samples that were not confirmed by SSCP.

Statistical analyses

Fisher exact, χ^2 , Mann-Whitney U, and unpaired *t* tests were used to analyze the association between clinicopathologic, genetic, and expression variables. Kaplan-Meier survival analysis and Mantel-Cox test were used to determine the progression-free survival of patients. Cox regression analysis was used to assess the independent prognostic value of study variables. In the regression-tree analysis, all study variables were included in the model. The strongest prognostic factor was used to divide the entire patient group into two subgroups. The analysis was then repeated in these subgroups to find which, if any, of the remaining variables would further divide the patients into smaller prognostically distinct groups. The process was continued as long as a significant ($P < 0.05$) progression-free survival difference was found between the two groups. Five-year progression-free survival was calculated for each branch for the regression-tree. For heterogeneity analyses of the metastases with quantitative immunohistochemistry (IHC) values, we computed SD between different metastases from a single individual. Empirical background distribution of SD values was generated by randomly selecting corresponding number of tumors across individuals. Random selection was repeated 1,000 times. *P* values correspond to the probability of observing a given or smaller SD value in the background distribution.

Results

Comparison of TMPRSS2:ERG fusion detection by FISH and ERG expression according to IHC

Altogether, 284 prostatectomy and locally recurrent CRPC samples were analyzed by both FISH and IHC. *TMPRSS2:ERG* fusion was seen in 87 of 143 (61%) ERG-positive samples, whereas 3 of 141 (2%) ERG-negative samples showed *TMPRSS2:ERG* fusion ($P < 0.0001$; Supplementary Table S7).

Prostatectomy specimens

Positive ERG staining (intensity 1–3) was seen in 48% (137/287) of the prostatectomy samples (Table 1; Supplementary Fig. S1A and S1B). The ERG positivity rate remained same regardless of the use of PSA in diagnosis. Samples obtained before the introduction of PSA measurements (diagnosis in 1980s), as well as samples from the time of occasional (diagnosis between 1990–1995) and routine PSA (diagnosis 1996–) tests showed ERG positivity in 45%, 48%, and 47% of the samples, respectively (Supplementary Table S8). ERG positivity was significantly associated with longer progression-free survival compared with ERG-negative cases in Kaplan-Meier analysis ($P = 0.0014$; Fig. 1A). If we excluded the cases included in our previously published analysis (6), the association still remained significant ($P = 0.0371$, Supplementary Fig. S2). In multivariate analysis, ERG showed independent prognostic value (Table 2). The regression-tree analysis demonstrated that the prognostic power of ERG was restricted to large tumors (pT3 stage) as well to smaller (pT2) tumors with high Gleason score (GS; Supplementary Fig. S3). High Ki-67 and EZH2 expression was significantly associated with ERG positivity ($P = 0.0034$, $P < 0.0001$, respectively; Table 1).

High nuclear AR staining (intensity 2–3) was seen in 82% (206/252) and low (intensity 1) or negative (intensity 0) in 18% (46/252) of the prostatectomy samples. 5% (13/252) of the samples showed no AR staining (Table 1; Supplementary Fig. S1C and S1D). High nuclear AR expression was associated with ERG positivity ($P < 0.0001$; Table 3). Interestingly, samples expressing low or no AR staining had significantly higher primary PSA levels compared with tumors with high AR expression ($P = 0.0276$). This association was lost when totally AR-negative tumors were compared with the rest of the prostatectomy samples ($P = 0.6030$). High AR expression was significantly associated with high Ki-67 and EZH2 expression ($P < 0.0001$, $P < 0.0001$, respectively; Table 1), but not with progression-free survival ($P = 0.3089$; Fig. 1B).

Negative PTEN staining (intensity 0) was seen in 15% (42/282) of the samples (Table 1; Supplementary Fig. S1E and S1F). ERG positivity was significantly associated with loss of PTEN expression ($P = 0.0348$; Table 3). Cases with no PTEN expression had significantly shorter progression-free survival than patients with some PTEN expression ($P = 0.0133$; Fig. 1C). In multivariate analysis, PTEN showed borderline independent prognostic value (Table 2). In ERG-positive cases, the progression-free survival was significantly shorter in PTEN negative compared with positive cases ($P = 0.0085$; Fig. 1D). In contrast, in ERG-negative cases, PTEN expression was not associated with the progression-free survival ($P = 0.5614$; Fig. 1E). In the regression-tree analysis, PTEN showed prognostic value in subgroups of tumors with pT2 stage, GS 7 and ERG positivity as well as in tumors with pT2, GS < 7 and 10 ng/mL < PSA ≤ 30 ng/mL (Supplementary

Table 1. Association of clinicopathological variables, and Ki-67 and EZH2 expression levels with expressions of ERG, AR, and PTEN

Variable	ERG expression		P	AR expression		P	PTEN expression		P
	Negative	Positive		Low	High		Negative	Positive	
Prostatectomy specimens, n (%)	150 (52)	137 (48)		46 (18)	206 (82)		42 (15)	240 (85)	
Needle biopsy specimens, n (%)	92 (55)	74 (45)		-	-		-	-	
Locally recurrent CRPCs, n (%)	81 (53)	72 (47)		9 (7)	126 (93)		55 (45)	67 (55)	
Metastasized CRPCs, n (%) ^a	17 (55)	14 (45)	0.9653	3 (10)	27 (90)	0.0434	19 (61)	12 (39)	<0.0001
Prostatectomy specimens									
Gleason score, n (%) ^a									
<7	51 (49)	53 (51)		14 (15)	80 (85)		13 (13)	91 (88)	
7	70 (50)	70 (50)		22 (18)	102 (82)		19 (14)	116 (86)	
>7	25 (71)	10 (29)	0.0530	8 (29)	20 (71)	0.2526	9 (24)	28 (76)	0.2076
pT Stage, n (%) ^b									
pT2	101 (52)	94 (48)		30 (18)	140 (82)		26 (14)	161 (86)	
pT3	46 (53)	41 (47)	0.8978	15 (19)	63 (81)	0.8593	16 (18)	74 (82)	0.4745
PSA ng/mL (mean ± SD) ^c	17.3 ± 23.4	12.6 ± 9.3	0.2321	17.7 ± 14.2	14.0 ± 19.0	0.0276	13.6 ± 11.0	15.7 ± 19.4	0.8874
Age (mean ± SD) ^d	62.8 ± 5.4	62.9 ± 4.9	0.8339	63.4 ± 4.4	62.6 ± 5.4	0.3377	63.1 ± 5.9	62.9 ± 5.1	0.9015
Ki-67 (mean ± SD) ^c	8.1 ± 10.2	12.5 ± 15.1	0.0034	4.9 ± 7.7	11.4 ± 13.6	<0.0001	13.7 ± 18.6	9.6 ± 11.7	0.2381
EZH2 (mean ± SD) ^c	29.6 ± 21.1	41.5 ± 24.9	<0.0001	21.8 ± 20.2	40.4 ± 23.8	<0.0001	35.9 ± 23.8	35.1 ± 23.4	0.8610
Needle biopsy specimens									
Gleason score, n (%) ^a									
<7	24 (62)	15 (38)		-	-		-	-	
7	23 (45)	28 (55)		-	-		-	-	
>7	43 (61)	27 (39)	0.1508	-	-	-	-	-	-
T Stage, n (%) ^b									
T1-2	51 (54)	44 (46)		-	-		-	-	
T3-4	41 (58)	30 (42)	0.6382	-	-	-	-	-	-
M stage, n (%) ^b									
M0	36 (60)	24 (40)		-	-		-	-	
M1	12 (52)	11 (48)	0.6212	-	-	-	-	-	-
PSA ng/mL (mean ± SD) ^c	6.7 ± 24.8	8.0 ± 25.8	0.9399	-	-	-	-	-	-
Age (mean ± SD) ^d	74.1 ± 6.9	73.1 ± 7.0	0.3892	-	-	-	-	-	-
Tumor area% (mean ± SD) ^e	29.1 ± 26.8	33.3 ± 28.8	0.3561	-	-	-	-	-	-
Ki-67 (mean ± SD) ^c	7.5 ± 6.1	8.7 ± 6.2	0.0874	-	-	-	-	-	-
EZH2 (mean ± SD) ^c	20.0 ± 18.2	22.3 ± 18.2	0.3327	-	-	-	-	-	-
Locally recurrent CRPCs									
Ki-67 (mean ± SD) ^c	17.2 ± 14.6	19.8 ± 15.7	0.3697	26.0 ± 28.0	17.5 ± 13.5	0.9766	21.1 ± 14.4	16.4 ± 14.5	0.0597
EZH2 (mean ± SD) ^c	57.5 ± 26.9	47.1 ± 27.9	0.0373	44.3 ± 34.3	54.3 ± 27.4	0.3513	61.6 ± 28.2	47.8 ± 26.9	0.0169
Metastasized CRPCs									
Ki-67 (mean ± SD) ^c	15.5 ± 20.2	17.2 ± 10.4	0.1536	28.4 ± 44.7	15.0 ± 10.9	0.5094	17.3 ± 19.1	14.9 ± 10.9	0.8324
EZH2 (mean ± SD) ^c	40.4 ± 29.9	40.3 ± 19.9	0.8297	43.7 ± 46.9	41.5 ± 22.7	0.9724	35.0 ± 26.4	48.9 ± 22.4	0.1406

^a χ^2 test.^bFisher's exact test.^cMann-Whitney U test.^dUnpaired *t* test.^eUnpaired *t* test with Welch correction.

Fig. S3). PTEN loss was also significantly associated with hemizygous deletion of *TP53* ($P = 0.0281$, Supplementary Table S9).

Strong SPINK1 expression (intensity 3) was found in 14% (34/243) of the samples (Table 4; Supplementary Fig. S1G and S1H) and almost exclusively in ERG-negative cases ($P = 0.0002$; Table 3). Strong SPINK1 staining was significantly associated with high Gleason score ($P = 0.0305$; Table 4). There was no significant difference in progression-free survival between high and low SPINK1

expression ($P = 0.2479$; Fig. 2A). In ERG-negative cases, SPINK1 did not either have prognostic value ($P = 0.9056$; Supplementary Fig. S4).

FISH was carried out to detect the frequency of *TP53* deletion (Supplementary Fig. S5A and S5B). Hemizygous deletion of *TP53* was found in 7% (17/234) of the cases (Table 4). There was no difference in ERG expression levels ($P = 0.2108$; Table 3) or in progression-free survival between cases with or without deletion ($P = 0.7746$; Fig. 2B). However, the deletion was associated with high pT

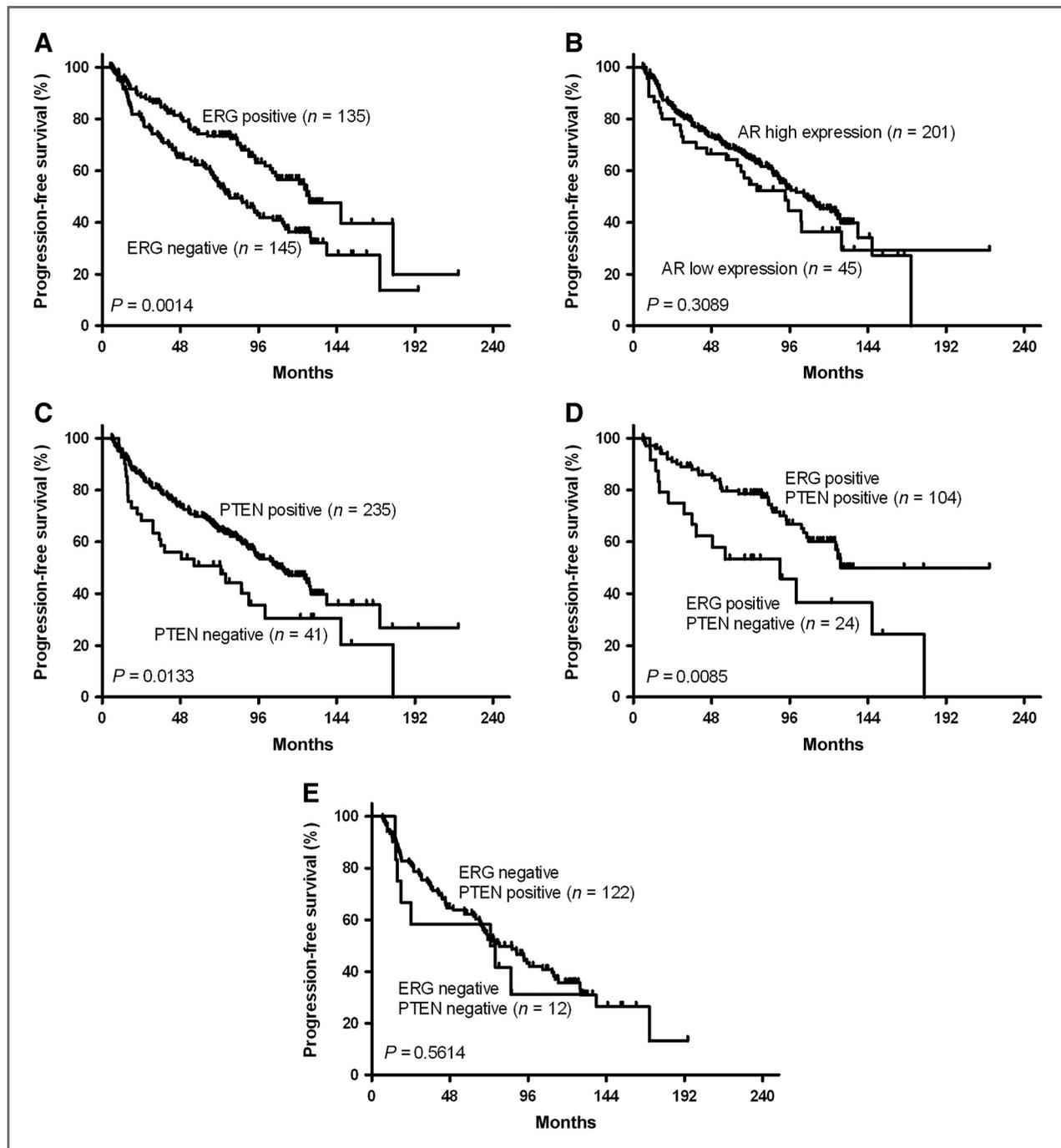


Figure 1. Kaplan-Meier analysis of progression-free survival of prostatectomy-treated patients according to (A) ERG, (B) AR, and (C) PTEN. D, ERG-positive patients with loss of PTEN expression had significantly shorter progression-free survival ($P = 0.0085$). E, PTEN expression had no significant effect on progression-free survival of ERG-negative patients ($P = 0.5614$). Mantel-Cox test was used to calculate the P values.

stage and low diagnostic PSA levels ($P = 0.0480$, $P = 0.0386$, respectively; Table 4).

FISH was also used to detect the frequency of chromosome 3p14 deletion (Supplementary Fig. S5C and S5D). Deletion of chromosomal region 3p14 was seen in 3% (6/229) of the specimens (Table 4). The expression of ERG was not associated with 3p14 deletion ($P = 0.4317$; Table 3).

Prostate needle biopsies

The biopsy cohort represented more advanced disease and older patients than the prostatectomy cohort. Positive ERG staining was seen in 45% (74/166) of the biopsies (Table 1). The ERG expression was not associated with progression-free survival (Supplementary Fig. S6), or Ki-67 and EZH2 expression levels (Table 1). Strong SPINK1

Table 2. Multivariate analysis of the variables of prostatectomy samples

Variable	RR (95% CI)	P
pT Stage (3 vs. 2)	2.59 (1.66–3.97)	<0.001
PSA (>30 vs. >10 and ≤30, vs. ≤10 ng/mL)	1.47 (1.10–1.97)	0.001
Gleason score (>7 vs. 7 vs. <7)	1.52 (1.09–2.11)	0.008
ERG (positive vs. negative)	0.59 (0.39–0.89)	0.019
PTEN (positive vs. negative)	0.57 (0.33–0.97)	0.052

NOTE: Other non-significant variables in the model: age.

Abbreviations: RR, relative risk; 95% CI, 95% confidence interval.

expression was seen in 18% (29/161) of the samples (Table 4), and it was found almost exclusively in ERG-negative samples (Table 3).

Locally recurrent CRPCs

Positive ERG staining was seen in 47% (72/153) of the locally recurrent CRPCs (Table 1). ERG negativity was associated with high EZH2 ($P = 0.0373$; Table 1), whereas not with Ki-67.

High nuclear AR expression was found in 93% (126/135) and low or negative expression in 7% (9/135) of the samples (Table 1). Two of 135 (1.5%) tumor samples were negative. ERG was not associated with AR expression levels (Table 3).

Negative PTEN expression was seen in 45% (55/122) and low or negative in 51% (62/122) of the cases (Table 1). ERG positivity was significantly associated with loss of PTEN expression ($P = 0.0042$; Table 3). Negative PTEN expression was associated with higher EZH2 expression level ($P = 0.0169$, Table 1).

Strong SPINK1 expression was found in 12% (17/137), deletion of *TP53* (hemizygous) in 18% (24/136), and deletion of chromosome 3p14 region in 4% (5/112) of the

cases (Table 4). Strong SPINK1 expression was seen almost exclusively in ERG negative cases ($P = 0.0058$, Table 3). ERG expression levels were not associated with deletions of *TP53* and 3p14 (Table 3).

CRPC metastases

One hundred and fourteen CRPC metastases from 32 study subjects were studied. Samples showing no immunoreactivity with any of the antibodies were excluded from analyses. Thus, one hundred metastases from 31 subjects were suitable for the analyses. Twenty-five subjects had 2 to 5 and the remaining 6 had one metastasis available. Positive ERG staining was seen in 49% (44/90) of the samples, and in 45% (14/31) of the subjects (Table 1). *TMPRSS2:ERG* fusion transcripts have been studied in a small subset of these subjects by Liu and Laitinen and colleagues (29). All cases with *TMPRSS2:ERG* fusion transcripts (6 samples) also showed positive ERG staining. ERG expression was not associated with Ki-67 or EZH2 staining (Table 1).

High nuclear AR expression was found in 76% (68/90) and low or negative in 24% (22/90) of the samples. Five of 90 (6%) samples were totally negative for AR. Of the 30,

Table 3. Association of ERG with AR, PTEN, and SPINK1 expression, and deletions of *TP53* and 3p14 region in prostatectomy, diagnostic needle biopsy, locally recurrent CRPC, and metastasized CRPC specimens

Variable	Prostatectomy specimens, n (%)		P^a	Needle biopsy specimens, n (%)		P^a	Locally recurrent CRPCs, n (%)		P^a	Metastasized CRPCs, n (%)		P^a
	ERG Negative	ERG Positive		ERG Negative	ERG Positive		ERG Negative	ERG Positive		ERG Negative	ERG Positive	
AR low expression	37 (31)	7 (6)		—	—		3 (4)	6 (11)		3 (19)	0 (0)	
AR high expression	84 (69)	114 (94)	<0.0001	—	—		65 (96)	50 (89)	0.2971	13 (81)	14 (100)	0.2276
PTEN negative	13 (9)	24 (18)		—	—		20 (32)	30 (60)		9 (53)	10 (71)	
PTEN positive	125 (91)	106 (82)	0.0348	—	—		42 (68)	20 (40)	0.0042	8 (47)	4 (29)	0.4607
SPINK1 low	89 (77)	107 (95)		63 (72)	69 (95)		53 (79)	55 (96)		11 (65)	8 (57)	
SPINK1 high	26 (23)	6 (5)	0.0002	25 (28)	4 (5)	0.0001	14 (21)	2 (4)	0.0058	6 (35)	6 (43)	0.7241
No <i>TP53</i> deletion	106 (95)	96 (90)		—	—		61 (86)	44 (76)		9 (100)	7 (78)	
<i>TP53</i> deletion	6 (5)	11 (10)	0.2108	—	—		10 (14)	14 (24)	0.1750	0 (0)	2 (22)	0.4706
No 3p14 deletion	111 (98)	101 (96)		—	—		62 (95)	46 (96)		—	—	
3p14 deletion	2 (2)	4 (4)	0.4317	—	—		3 (5)	2 (4)	1.0000	—	—	

^aFisher's exact test.

Table 4. Association of clinicopathologic variables and Ki-67 and EZH2 expression levels with expression of SPINK1 and deletions of *TP53* and 3p14 region

Variable	SPINK1 expression		P	TP53 deletion		P	3p14 deletion		P
	Low	High		No deletion	Deletion		No deletion	Deletion	
Prostatectomy specimens, n (%)	209 (86)	34 (14)		217 (93)	17 (7)		223 (97)	6 (3)	
Needle biopsy specimens, n (%)	132 (82)	29 (18)							
Locally recurrent CRPCs, n (%)	120 (88)	17 (12)		112 (82)	24 (18)		107 (96)	5 (4)	
Metastasized CRPCs, n (%) ^a	19 (61)	12 (39)	<0.0001	17 (89)	2 (11)	0.0531	13 (93)	1 (7)	0.3776
Prostatectomy specimens									
Gleason score, n (%) ^a									
<7	88 (93)	7 (7)		81 (89)	10 (11)		87 (100)	0 (0)	
7	93 (80)	23 (20)		105 (95)	5 (5)		110 (97)	3 (3)	
>7	24 (89)	3 (11)	0.0305	25 (96)	1 (4)	0.1640	21 (95)	1 (5)	0.2235
pT Stage, n (%) ^b									
pT2	143 (87)	21 (13)		154 (95)	8 (5)		154 (97)	4 (3)	
pT3	63 (84)	12 (16)	0.7482	58 (87)	9 (13)	0.0480	63 (98)	1 (2)	1.0000
PSA ng/mL (mean ± SD) ^c	13.2 ± 10.7	21.5 ± 40.4	0.2749	13.6 ± 10.8	9.3 ± 7.6	0.0386	13.5 ± 18.2	11.7 ± 3.1	0.5077
Age (mean ± SD) ^d	62.9 ± 5.1	63.1 ± 4.8	0.7714	63.0 ± 5.0	60.8 ± 6.2	0.1042	62.5 ± 4.9	67.5 ± 4.5	0.0159
Ki-67 (mean ± SD) ^c	9.3 ± 12.0	10.1 ± 12.5	0.8064	10.1 ± 12.8	10.0 ± 7.8	0.2298	10.1 ± 12.1	22.2 ± 23.3	0.3359
EZH2 (mean ± SD) ^c	36.8 ± 23.4	33.0 ± 24.5	0.3166	36.4 ± 24.6	35.9 ± 20.6	0.9658	37.5 ± 24.0	49.8 ± 28.0	0.2534
Locally recurrent CRPCs									
Ki-67 (mean ± SD) ^c	18.2 ± 15.0	16.1 ± 8.7	0.9835	17.9 ± 16.5	20.5 ± 10.8	0.0871	18.7 ± 16.1	16.3 ± 7.8	0.9359
EZH2 (mean ± SD) ^c	52.2 ± 27.8	64.9 ± 25.1	0.1041	49.5 ± 28.7	60.9 ± 26.8	0.0745	51.6 ± 27.9	65.7 ± 26.6	0.2930
Metastasized CRPCs									
Ki-67 (mean ± SD) ^c	13.6 ± 8.0	18.1 ± 19.8	0.9325	13.5 ± 10.4	12.3 ± 3.3	0.9412	-	-	
EZH2 (mean ± SD) ^c	41.5 ± 27.4	39.8 ± 25.1	0.7256	42.1 ± 18.7	35.9 ± 12.7	0.9412	-	-	

^aχ² test.^bFisher's exact test.^cMann-Whitney U test.^dUnpaired t test.

27 (90%) showed high AR expression in at least one tumor sample, whereas 2 subjects (7%) had totally negative AR expression in all tumor samples (Table 1). AR expression level was not associated with the ERG positivity (Table 3).

Loss of PTEN expression was found in 67% (61/91) of the samples. Of the subjects, 61% (19/31) showed loss of PTEN expression (Table 1). Homozygous *PTEN* deletion has been previously reported in subjects A28, A4, and A7 (29, 30). PTEN staining was negative in all of these subjects' samples. Loss of heterozygosity has been previously reported in subjects A5, A8, A10, A13, A14, and A16 (28). Four of these subjects showed negative PTEN staining in all of their samples. Loss of PTEN expression was not associated with ERG expression, although there was a trend (Table 3).

Strong SPINK1 expression was seen in 14% (13/91) of all samples. Of the subjects, 39% (12/31) showed strong SPINK1 expression in, at least, one sample (Table 4). Strong SPINK1 expression was not associated with ERG expression levels (Table 3).

Hemizygous deletion of *TP53* was seen in 7 of 46 (15%) samples. The seven deletions were from two subjects. Thus, of the 19 informative study subjects two (11%) showed deletion of *TP53* in at least one tumor sample

(Table 4). *TP53* deletion was not associated with ERG expression levels (Table 3).

Deletion of chromosomal region 3p14 was seen in 2 of 41 (5%) samples. These two deletion cases were from the same subject. Thus, of the 14 informative study subjects one (7%) showed deletion of region 3p14 (Table 4). Because of the low frequency of chromosome 3p14 deletions, no further studies were conducted with this aberration.

Mutation analysis of *TP53* in CRPC metastases

A *TP53* mutation was found in 37% (36/98) of all metastases, and among the subjects, 41% (13/32) showed mutation at least in one sample (Supplementary Table S10). In 12 of 13 cases, identical *TP53* mutations were found among all metastatic sites studied. Subject A5 was the only case where nonuniform *TP53* mutation was found. Two different mutations were found in separate metastases, and one metastasis did not have any *TP53* mutation. No *TP53* mutation was found in noncancerous control samples studied in parallel from each subject. Of the 14 mutations identified, 10 were substitutions. Of these, one was a missense substitution in exon 4, three were missense substitutions in exon 5, two were chain-terminating substitutions in exon 6, and four were

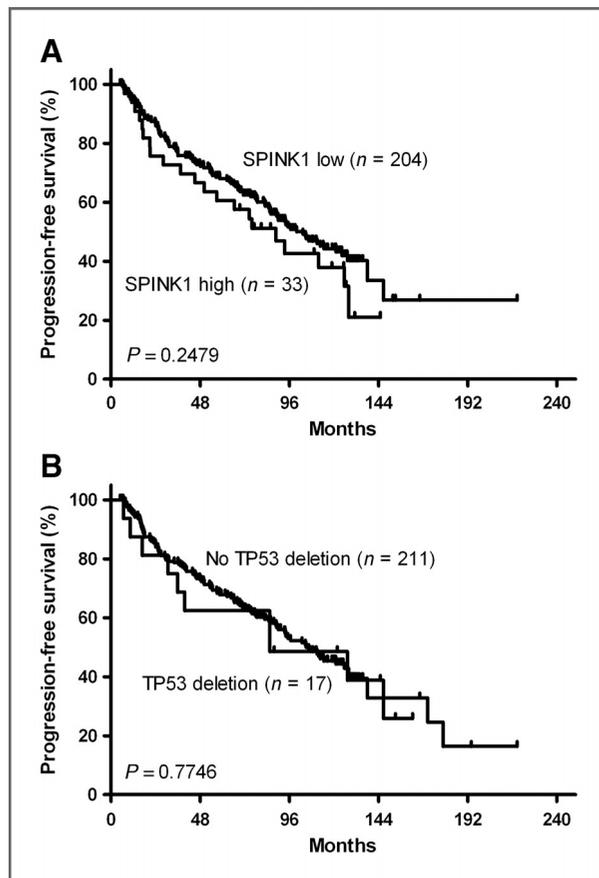


Figure 2. Kaplan-Meier analysis of progression-free survival of prostatectomy-treated patients according to (A) SPINK1 expression, and (B) TP53 deletion. Mantel-Cox test was used to calculate the *P* values.

missense substitutions in exon 8. Three of the mutations were deletions. These were a 14 base pair (bp) deletion in intron 5, a 14 bp deletion in exon 6, and a 2 bp deletion in exon 6. One of the identified mutations was a one bp insertion in exon 7. All deletions (except the deletion in intron 5) and insertion mutations caused a frameshift, leading to an aberrant protein product or a chain termination signal. TP53 alterations (mutation or deletion) tended to be more common in ERG-positive than negative subjects ($P = 0.0720$; Supplementary Table S11).

Comparison of prostatectomy, needle biopsy, locally recurrent CRPC, and metastasized CRPC specimens

The frequency of ERG expression was very similar in the 4 sample types, ranging from 45% to 48% (Table 1). CRPC tumors showed higher AR expression than the hormone-naïve prostatectomy specimens ($P = 0.0434$; Table 1). Loss of PTEN expression was also significantly more frequent in CRPC than in prostatectomy specimens ($P < 0.0001$; Table 1). The frequency of strong SPINK1 expression was significantly higher in CRPC metastases than in the rest of the sample sets studied ($P < 0.0001$; Table 4). TP53 deletion tended to be more frequent in CRPC than

in hormone-naïve tumors ($P = 0.0531$; Table 4). Deletion of 3p14 was rare in all tumor types.

Heterogeneity of CRPC metastases

We studied the heterogeneity of the immunostaining and TP53 mutations between metastases from each subject with at least two different metastatic sites. Criteria for positive and negative or low and high expression were as before (Supplementary Table S4). Consistent, either positive or negative, ERG expression was seen in 20 of 24 (83%) subjects. Eight showed positive and 12 negative expression. Consistent PTEN expression was seen in 19 of 24 (79%) subjects, of which 14 showed loss of PTEN expression, and 5 no loss. Identical TP53 mutations were found in metastases from 12 of 13 (92%) subjects. When low (intensity 0 to 1) and high (intensity 2 to 3) expression criteria were used for AR, 14 of 25 (56%) subjects showed consistent results between the metastases. However, if AR expression was classified as positive (intensity 1 to 3) or negative (intensity 0) consistent results were seen in all subjects. Four metastatic samples from the same subject were totally negative for AR. Of the subjects showing strong SPINK1 expression, none had consistent SPINK1 staining in all metastases. For the Ki-67 and EZH2 quantitative IHC values, we compared the SD of different metastases compared with that of randomly selected metastases from whole cohort. The *P* value indicated that in 5 of 12 informative (>3 metastases per subject) cases, the SD of EZH2 staining was significantly ($P < 0.05$) smaller between the metastases from the same subject compared with randomly selected metastases. Similarly, 1 of 10 cases showed a smaller SD for Ki-67 staining in the subject compared with randomly selected metastases.

Discussion

In agreement with previous reports (6, 31, 32), we observed positive ERG staining in 45%–48% of prostatectomy, prostate needle biopsy, locally recurrent CRPC and metastasized CRPC samples, respectively (Fig. 3). We have previously reported that *TMPRSS2:ERG* fusion is associated with longer progression-free survival in prostatectomy-treated patients (6). Here, we almost doubled the sample size, and examined ERG protein expression instead of fusion, and again, ERG positivity was significantly associated with longer progression-free survival in prostatectomy-treated patients. We also compared the ERG expression in our previously published prostatectomy material (6) with the new cases. In both sample sets, Kaplan-Meier survival curves demonstrated longer progression-free survival for ERG-positive cases. Even in pT3 (non-organ confined) primary prostate cancer, the fraction of patients with 5-year progression-free survival is higher when ERG status is positive. This further suggests that the molecular mechanisms of aggressiveness are not related ERG status *per se*. Most of the published studies have not found an association between ERG and prognosis (13). The discrepancy may be due to differences

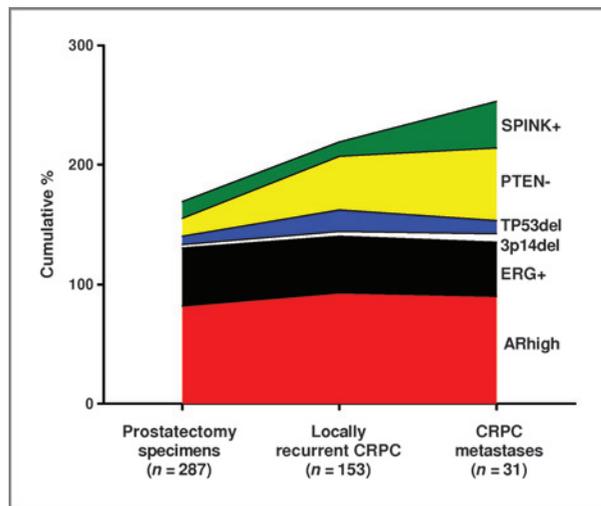


Figure 3. Cumulative percentage of ERG positivity, high expression of AR, loss of PTEN, deletion of TP53 and 3p14, and strong SPINK1 staining in prostatectomy, locally recurrent CRPC, and CRPC metastasis specimens.

between the patient cohorts studied. Our prostatectomy material consists of population-based consecutive cases from a single institution with patients accrued both before and after the PSA era. Notably, the ERG positivity rate was similar in primary cancers detected before and after the onset of routine use of PSA.

One caveat in our material was that we could analyze only progression-free, in practice biochemical (i.e., PSA) progression-free survival, and not prostate cancer-specific survival. It has recently been shown that ERG may suppress the expression of PSA through EZH2 and HDACs (33). Thus, PSA might not be a good surrogate marker of prognosis in the context of ERG. The association of ERG expression with good prognosis is contradictory to the finding of a strong correlation with high Ki-67 and EZH2 stainings. Both Ki-67 and EZH2 being markers of poor prognosis in prostate cancer (34).

We and others (14, 15) have previously reported that the *TMPRSS2:ERG* fusion is not associated with prognosis in primarily endocrine treated patients. Here, we confirm this finding by showing that the ERG expression is not associated with prognosis in endocrine-treated patients. The discrepancy between the prostatectomy and endocrine-treated cohorts could be due to the fact that the latter one represents more advanced disease.

The genes and proteins that we studied were selected because they have previously been suggested to be associated with ERG. Indeed, we were able to confirm some of the associations. For example, our data on prostatectomy samples are in concordance with the study by Yu and colleagues (17), who showed that expression of ERG and EZH2 are positively correlated, and that ERG can activate the expression of EZH2. However, we did not find similar association in the biopsy or CRPC samples. Actually, in locally recurrent CRPCs, EZH2 expression was marginally higher in ERG-negative than positive cases. CRPC

tumors had higher average expression of EZH2 than prostatectomy or needle biopsy specimens. The findings may suggest that ERG regulates EZH2 only in early disease, whereas other mechanisms regulate EZH2 expression in advanced disease or CRPC.

The expression of AR was significantly higher in CRPC than in the hormone-naïve prostate cancer specimens as has been previously reported (35–37). In prostatectomy samples, high AR expression was significantly associated with higher Ki-67 and EZH2 expression levels. *In vitro* studies have shown that AR increases ERG expression in *TMPRSS2:ERG* fusion positive cells (16). In clinical prostate cancer samples, positive and negative correlations have been found between AR and ERG (17, 32). In this study, high nuclear AR expression was significantly associated with ERG expression in prostatectomy, but not in the CRPC samples. This can be explained by the high overall AR expression in CRPC samples.

PTEN is a commonly altered tumor suppressor gene in prostate cancer, but mainly through copy number loss rather than point mutation (18). Hemizygous *PTEN* deletion is found in approximately 24%–39% and homozygous in 5% of the prostatectomy specimens (18, 38). We found loss of *PTEN* expression in 15%, 45%, and 61% of prostatectomy, locally recurrent and metastasized CRPC samples, respectively (Fig. 3). In prostatectomy samples, loss of *PTEN* expression was significantly associated with shorter progression-free survival. This is in line with previous results suggesting a significant role for *PTEN* in prostate cancer progression (39, 40). The *TMPRSS2:ERG* fusion has previously been shown to be associated with deletion of *PTEN* and also with *PTEN* expression levels (18, 19, 41). Our study confirms the association in prostatectomy and CRPC samples. Also, absence of *PTEN* expression was associated with adverse progression-free survival in ERG-positive cases, but not in ERG-negative cases (Fig. 2). The data suggest interplay between ERG and *PTEN* pathways. These results are in line with previous report by Yoshimoto and colleagues (42). In contrast, Krohn and colleagues (43) reported that deletion of *PTEN* had prognostic impact on both ERG negative and positive prostate cancer cases, and Reid *et al.* (44) found that the deletion decrease prostate cancer-specific survival of patients with ERG/*ETV1* non-rearranged tumors.

The frequency of SPINK1 positivity (~10%) in local disease, either hormone-naïve or CRPC was about the same as we and others have previously found in hormone-naïve prostatectomy and needle biopsy specimens (14, 20). In prostatectomy-treated patients, contradictory results have been published concerning the effects of SPINK1 expression in progression and survival. Tomlins and colleagues, (20) found an association between positive SPINK1 expression and shorter recurrence-free time, whereas we have previously reported no such association (14). Here, with larger cohort we were able to replicate our previous finding. It has

previously been reported that SPINK1 overexpression is exclusive to ETS-fusion negative tumors in prostatectomy-treated samples (20). Our results confirmed that strong SPINK1 expression is found almost exclusively in ERG-negative samples in all other samples types except in metastases. As the frequency of SPINK1 expression in the CRPC metastases was higher than in the other specimens (Fig. 3), it may be that SPINK1 is involved especially in the processes of metastasis. And as ERG is not associated with metastasis, the association between ERG and SPINK1 is lost in the metastases.

Hemizygous deletion of *TP53* was seen in 7%, 18%, and 11% of prostatectomy, locally recurrent and metastasized CRPC samples, respectively (Fig. 3). The frequency of the deletion is lower than we have previously published in metastases (29), most likely due to poorer resolution of FISH compared with Affymetrix array 6.0 analysis. *TMPRSS2:ERG* fusion and positive ERG expression have previously been thought to be associated with deletion of *TP53* and p53 expression levels (18, 31). However, we did not find an association between ERG expression and *TP53* deletion in any of the sample sets. *TP53* mutation was found in 41% of the CRPC metastases, which is in line with previous studies (45, 46).

We used three-color FISH to detect the deletion of 3p14 region in 3% to 7% of cases (Fig. 3). The frequency is lower than that of 19% reported by Taylor and colleagues (18). This could be because our approach could not detect the smallest deletions in this area. *TMPRSS2:ERG* fusion has been shown to be associated with this deletion (18). However, we did not find any association between ERG expression and the deletion.

Our previous report from the same CRPC metastases as here showed that copy number variations among metastases in a subject are shared suggesting that they originate from the same primary tumor (29). The *TP53* mutation data here support the notion of a monoclonal origin of the metastases. We also showed here that immunostainings of ERG, PTEN, and AR were very homogenous among the metastases in a given subject. Thus, it seems that the level of expression of these genes is independent of local microenvironment. This is not surprising, since the expression of these genes is, at least partly, dictated by genetic alterations, for example for ERG, fusion with *TMPRSS2*, for PTEN, gene deletion, and for AR, gene amplification. In contrast, the immunostaining of SPINK1, Ki-67, and partly also EZH2, showed variability among the metastases in a given subject. Their variable expression levels may reflect adaptation to different microenvironments affecting to the metastases. Similar heterogeneity between metastases in the gene expression levels has also been reported previously (47).

The ERG aberrations were analyzed both for the expression with IHC as well as for the *TMPRSS2:ERG* fusion with FISH. Previous studies have demonstrated high concordance between the FISH and IHC methods (29, 48). Here, the concordance was 80%. Only 61% of

positive ERG samples by IHC had also detectable *TMPRSS2:ERG* fusion. This could be due to poor sensitivity of FISH or ERG fusion with other 5' fusion partners, such as *SLC45A3* and *NDRG1* (5). Three cases with *TMPRSS2:ERG* fusion in the absence of ERG expression could be due to failure of the staining.

In conclusion, our data show that the frequency of loss of PTEN expression, deletion of *TP53*, as well as high expression of SPINK1 increases from hormone-naïve localized and advanced disease to localized and metastasized CRPC, whereas ERG positivity do not (Fig. 3). Taking together that ERG expression is highly constant across the spectrum of pathologic stages and that it is not associated with adverse prognosis, it seems that ERG does not seem to be involved in the regulation of aggressiveness of prostate cancer. The association of ERG with the high expression of AR and EZH2 as well as loss of expression of PTEN suggests that these molecular pathways are interlinked. Especially, the finding that the prognostic value of PTEN loss is restricted to the ERG-positive cases indicates interaction between these two genetic aberrations. The data also support our previous finding that CRPC metastases in most of the subjects have common clonal origins.

Disclosure of Potential Conflicts of Interest

S. Egawa has a commercial research grant from Takeda Pharma Research fund, Astellas research fund, and AstraZeneca research fund. No potential conflicts of interest were disclosed by the other authors.

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