

Association of SPINK1 Expression and *TMPRSS2:ERG* Fusion with Prognosis in Endocrine-Treated Prostate Cancer

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Abstract

Purpose: The aim of the study was to examine whether *TMPRSS2:ERG* fusion or SPINK1 protein expression is associated with hormone responsiveness of prostate cancer and can thus be used as a biomarker.

Experimental Design: Diagnostic needle biopsies from prostate cancer patients primarily treated by endocrine therapy were evaluated for *TMPRSS2:ERG* fusion with fluorescence *in situ* hybridization and SPINK1 protein expression with immunohistochemistry.

Results: The frequency of *TMPRSS2:ERG* fusion in 178 biopsies of hormonally treated patients was 34%. Of the fusion-positive cases, 71% showed deletion between the two genes, and 23% showed gain of the fusion. The fusion was associated with high Ki-67 staining ($P = 0.001$), age at diagnosis ($P = 0.024$), and tumor area ($P = 0.006$), but not with Gleason score, T stage, M stage, prostate-specific antigen (PSA), or progression-free survival. Strong positive SPINK1 expression was found in 11% (21 of 186) of the biopsies. SPINK1-positive cases had significantly shorter progression-free survival compared with SPINK1-negative cases ($P = 0.001$). The expression was not associated with any other clinicopathologic variables studied. In a multivariate analysis, SPINK1 expression showed independent prognostic value, with a relative risk of 2.3 (95% confidence interval, 1.1-4.6). SPINK1 expression and the fusion were not associated with each other.

Conclusions: There was no association between *TMPRSS2:ERG* fusion and prognosis, suggesting that *TMPRSS2:ERG* rearrangement does not implicate hormone dependence of the cancer. SPINK1 expression, found in ~10% of prostate cancers, was associated with aggressive form of the disease and could serve as a biomarker in endocrine-treated prostate cancer. *Clin Cancer Res*; 16(10); 2845-51. ©2010 AACR.

It has been shown that gene fusions affecting ets transcription factors are common in prostate cancer (1). The most common type of the fusion (~85% of all fusion-positive cases) occurs between the androgen-regulated, prostate-specific *TMPRSS2* (transmembrane protease, serine 2) and the ETS family transcription factor *ERG* [v-ets erythroblastosis virus E26 oncogene like (avian); ref. 2], which are located 2.8 Mb apart on chromosome 21, in the same 5'-to-3' end orientation. The fusion can occur through interstitial deletion or through translocation. Deletion is thought to be more common (3-5). More than 20 different *TMPRSS2:ERG* fusion transcripts have already

been found (2-4, 6-9). The most common fusion isoform consists of exon 1 of *TMPRSS2* fused to exon 4 of *ERG* (2). Some fusion isoforms have been associated with more aggressive prostate cancer than others, bringing more diversity to the fusion-positive cases (6, 7). The expression of *TMPRSS2:ERG* fusion transcripts is regulated by androgens (1) and only found in androgen receptor-positive tumors (4, 10). Tumors containing the fusion, but not expressing androgen receptors, do not express the fusion transcript (4, 10).

The expression of SPINK1, alias pancreatic secretory trypsin inhibitor or tumor-associated trypsin inhibitor (11), has been found to be increased in prostate cancer (12). Recently, Tomlins et al. (13) showed that SPINK1 is exclusively expressed in tumors without the *TMPRSS2:ERG* fusion. The data also indicated that SPINK1 is associated with poor prognosis in prostatectomy-treated patients. The authors suggested (13) that the fusion and SPINK1 identify separate mechanisms of prostate cancer progression.

Because the expression of *TMPRSS2:ERG* fusion gene is tightly controlled by androgens, we speculated that the fusion could be associated with androgen responsiveness of prostate cancer. Thus, we studied whether the fusion is associated with response to hormonal therapy. In addition to *TMPRSS2:ERG* fusion, we also studied the expression of SPINK1. As far as we know, this is the first study to analyze

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Translational Relevance

A large fraction of prostate cancer patients are treated by endocrine therapy. The response to the treatments varies considerably. Due to the development of other treatment modalities, such as chemotherapy (e.g., docetaxel), it would be important to be able to estimate a patient's response to endocrine treatment. Here, we show that SPINK1 expression is associated with poor prognosis in primarily hormonally treated patients, suggesting that SPINK1 could be used to predict treatment response. On the other hand, *TMPRSS2:ERG* fusion, which has been suggested to be a biomarker of aggressive disease, was not found to be a prognostic marker in this patient cohort.

the prognostic value of SPINK1 expression in primarily endocrine-treated prostate cancer.

Materials and Methods

Clinical tumor samples. The use of clinical material was approved by the ethical committee of the Tampere University Hospital and the National Authority for

Medicolegal Affairs. The retrospective material consisted of 196 diagnostic, formalin-fixed paraffin-embedded prostate needle biopsies. They were selected from 298 new prostate cancer cases that were primarily hormonally treated in the Tampere University Hospital during 1999 to 2003. The selection was based on the availability of representative (i.e., cancer-containing) tumor blocks. The needle biopsies had been fixed in neutral formalin and embedded in paraffin according to routine practice in Tampere University Hospital. The forms of the therapy were: orchiectomy, 41 cases; luteinizing hormone-releasing hormone (LHRH) analogue, 131 cases; bicalutamide, 21 cases; and maximal androgen blockade, 3 cases. The mean (\pm SD) time from diagnosis to the beginning of treatment was 1.0 ± 0.8 months. The patient files, including clinical and pathologic information, were obtained from Tampere University Hospital and local health centers of the Pirkanmaa Hospital District. The patients were followed until February 2009. The median (progression) follow-up time was 70 months (range, 2-118 mo). Progression was defined as an increase in PSA values in two consecutive measurements by 25% above nadir and an absolute increase of ≥ 2 ng/mL above nadir (14), or emergence of new metastases. There were 65 cases of progression and 12 prostate cancer-specific deaths. The type of endocrine therapy was not associated with T stage ($P = 0.4756$; χ^2 test), Gleason score ($P = 0.1171$; χ^2 test), or progression-free

Table 1. Association of clinicopathologic variables with *TMPRSS2:ERG* fusion and SPINK1 expression

Variable	<i>TMPRSS2:ERG</i> fusion according to FISH (%)		<i>P</i>	SPINK1 expression according to immunohistochemistry (%)*		<i>P</i>
	No fusion	Fusion		Negative	Positive	
All samples	117 (66)	61 (34)		165 (89)	21 (11)	
Gleason score, <i>n</i> (%) [†]						
<7	34 (76)	11 (24)		41 (85)	7 (15)	
7	34 (65)	18 (35)		50 (93)	4 (7)	
>7	45 (60)	30 (40)	0.2206	68 (87)	10 (13)	0.4849
T stage, <i>n</i> (%) [†]						
T ₁₋₂	74 (70)	31 (30)		99 (91)	10 (9)	
T ₃₋₄	43 (60)	30 (40)	0.1096	66 (86)	11 (14)	0.2780
M stage, <i>n</i> (%) [‡]						
M ₀	42 (66)	22 (34)		57 (88)	8 (12)	
M ₁	13 (56)	10 (44)	0.4593	8 (67)	4 (33)	0.4391
PSA ng/mL (mean \pm SD) [§]	206 \pm 1092	78 \pm 216	0.3641	105 \pm 429	63 \pm 104	0.6528
Ki-67% (mean \pm SD)	6.6 \pm 5.3	9.8 \pm 6.9	0.0010	7.6 \pm 6.1	7.8 \pm 5.1	0.6618
Age (mean \pm SD) [§]	74.3 \pm 7.2	71.9 \pm 5.9	0.0243	73.8 \pm 6.8	71.6 \pm 8.4	0.1728
Tumor area % (mean \pm SD)	24.3 \pm 24.2	37.1 \pm 29.9	0.0059	28.6 \pm 26.6	33.7 \pm 31.1	0.4252

*SPINK1 expression classified as positive when the intensity was 3 and the surface area stained was >10%.

[†] χ^2 test.

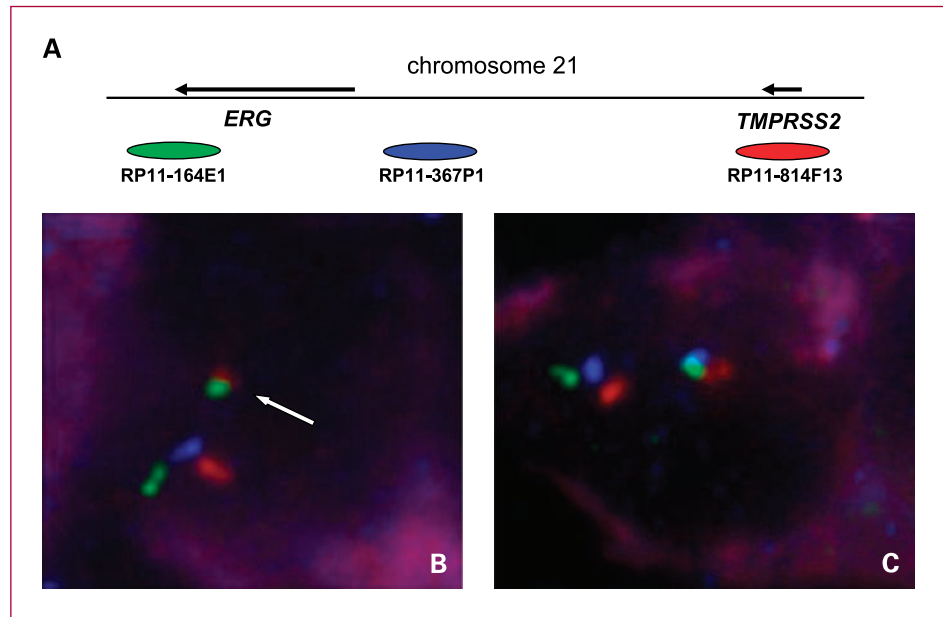
[‡]Fisher's exact test.

[§]Variance analysis.

^{||}Mann-Whitney test.

[¶]Variance analysis with Welch correction.

Fig. 1. A, the location of bacterial artificial chromosome probes used in the FISH assay on chr 21q22. Arrows, the chromosomal location and transcriptional direction of the genes; ovals, the probes in the colors seen in the FISH assay. B, three-color FISH analysis of a *TMPRSS2:ERG* fusion–positive case. The interstitial deletion is indicated by loss of the blue signal, and a yellowish signal indicates fusion (arrow). C, *TMPRSS2:ERG* fusion–negative specimen.



survival ($P = 0.5551$; Mantel-Cox test). The prostate cancer biopsy specimens were regraded for Gleason score by a pathologist (T.T. Tolonen). The clinicopathologic variables of the cases are shown in Table 1.

Immunohistochemistry. Antibodies against SPINK1 (6E8; ref. 15; U-H. S.) and Ki-67 (MM1; Novocastra Laboratories Ltd.) were used with Power Vision+ Poly-HRP IHC kit (ImmunoVision Technologies Co.) according to the manufacturer's instructions. Briefly, sections were deparaffinized and, for pretreatment, autoclaved in salt solution (5 mmol/L Tris-HCl/ 1 mmol/L EDTA; pH 9) at 98°C for 15 minutes. The primary antibody was diluted in preblocking solution and incubated overnight. The bound primary antibody was visualized with the use of a Lab Vision Autostainer. Sections were counterstained with hematoxylin and mounted with Neo-Mount (Merck). Every staining batch had a negative control, in which the primary antibody was omitted, and a positive control (formalin-fixed paraffin-embedded pancreatic tissue). Slides were scanned with an Aperio ScanScope XT scanner (Aperio Technologies, Inc.), and scoring was done blinded to the study end point with the use of a virtual microscope (<http://jvsmicroscope.uta.fi>) by one of the authors (T.T. Tolonen). SPINK1 staining was evaluated on a scale from 0 to 3, and the stained surface area was calculated. For scoring the Ki-67 staining, the images were analyzed in ImageJ. Briefly, the immunostained and counterstained nuclei were discriminated from the background and segmented, and the relative percentages of the nuclei were calculated.

Fluorescence in situ hybridization. Three-color fluorescence *in situ* hybridization (FISH) was carried out on 4-to-5- μ m-thick sections of the prostate cancer needle biopsy samples. Locus-specific bacterial artificial chro-

sosome probes for *ERG* (RP11-164E1), *TMPRSS2* (RP11-814F13), and the region between the two (RP11-367P1) were labeled with digoxigenin-dUTP (Roche Applied Science), Alexa Fluor 594-dUTP (Molecular Probes/Invitrogen), and biotin-dUTP (Roche Applied Science), respectively, by nick translation. To ensure the specificity and hybridization efficiency of the probes, hybridizations to normal human lymphocyte metaphase preparations were done. Deparaffinized slides were pretreated in 1 mol/L NaSCN solution for 10 minutes at 80°C, rinsed in water, and incubated in 4 mg/mL pepsin (Sigma-Aldrich Co.) in 0.9% NaCl (pH 1.5) for 25 minutes at 37°C. After washing and dehydration, the hybridization mixture containing the probes was applied to the slides, codenatured at 80°C for 10 minutes, and hybridized for 2 to 3 days at 37°C. After washes, slides were stained with anti-digoxigenin-FITC (Vector Laboratories) and streptavidin-Pacific Blue (Invitrogen), and counterstained with an antifade solution (Vectashield, Vector Laboratories) containing 0.1 mmol/L 4',6-diamidino-2-phenylindole. FISH signals were scored blinded to the study end point from non-overlapping nuclei with an Olympus BX50 epifluorescence microscope equipped with a charge-coupled device camera. Image-Pro Plus 6.1 software (Media Cybernetics Inc.) was used to capture stacks of nine images through each filter set, and the stacks were combined to produce a RGB image with an extended depth of focus.

Statistical analyses. Mann-Whitney U , χ^2 , and Fisher's exact tests, as well as variance analysis with Welch correction were used to analyze the association between clinicopathologic variables and both *TMPRSS2:ERG* fusion and SPINK1 expression. Kaplan-Meier survival analysis and Mantel-Cox test were used to determine the

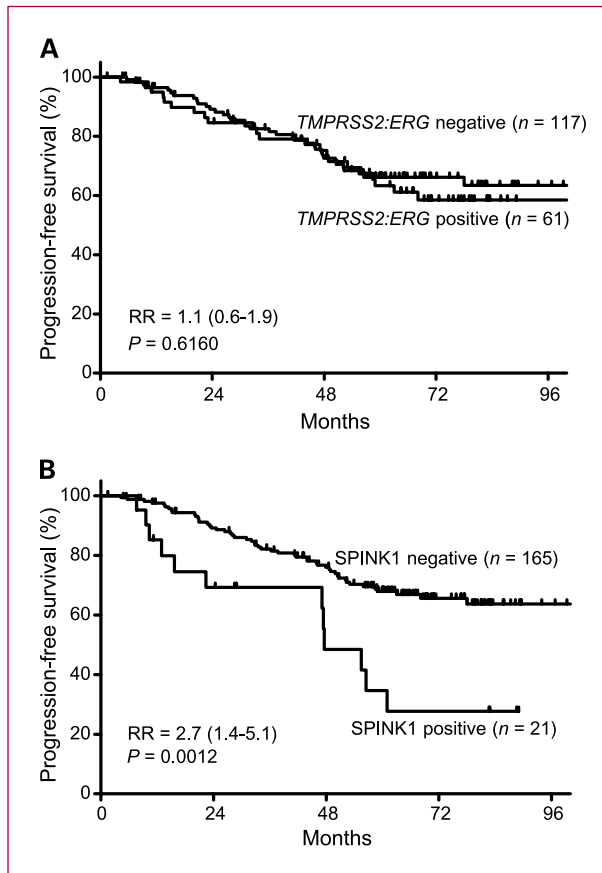


Fig. 2. Kaplan-Meier analyses of progression-free survival in endocrine-treated patients. A, *TMPRSS2:ERG* fusion-positive and *TMPRSS2:ERG* fusion-negative patients had no statistically significant difference in progression-free survival. B, SPINK1 expression-positive patients had significantly worse progression-free survival than SPINK1 expression-negative patients. Relative risk (RR) with 95% confidence interval according to Cox univariate analysis and *P*-value according to Mantel-Cox test are given.

progression-free survival of patients having *TMPRSS2:ERG* rearrangement or SPINK1 expression. Multivariate Cox regression analysis was done to estimate the independence of the prognostic markers.

Results

To assess the frequency of *TMPRSS2:ERG* fusions in 196 prostate cancer biopsy specimens, three-color FISH was carried out. The positions of the probes are shown in Fig. 1A. Of the 196 hybridized samples, 178 (91%) showed detectable FISH signals. *TMPRSS2:ERG* rearrangement was seen in 61 of 178 (34%) specimens, and of these, 43 of 61 (71%) showed deletion between the two genes. Fourteen of 61 (23%) fusion-positive cases showed gain of the fusion. Figure 1B shows an example of a specimen carrying *TMPRSS2:ERG* fusion through deletion. The fusion was not associated with Gleason score, T stage, M stage, and diagnostic PSA (Table 1). However, the fusion

was associated with Ki-67 staining ($P = 0.0010$), age at diagnosis ($P = 0.0243$), and tumor area ($P = 0.0059$). Thirty-six percent (22 of 61) of fusion-positive and 31% (36 of 117) of fusion-negative patients experienced progression during the follow-up time. There was no difference in the progression-free survival of the *TMPRSS2:ERG* fusion-positive and *TMPRSS2:ERG* fusion-negative cases ($P = 0.6160$; Fig. 2A). When gain of *TMPRSS2:ERG* or deletion/translocation of *TMPRSS2:ERG* was studied separately, it did not show an association with prognosis either.

SPINK1 protein expression in 196 hormone-treated prostate cancer specimens was determined by immunohistochemistry. Staining was successful in 186 of 196 (95%) samples. Figure 3 shows SPINK1-positive and SPINK1-negative staining. The criterion for positive SPINK1 expression was defined as >10% of the cancerous tissue stained with intensity 3. Positive SPINK1 expression was seen in 21 of 186 (11%) specimens, in which the stained surface area varied from 20% to 100%. SPINK1 expression was not associated with Gleason score, T stage, M stage, diagnostic PSA, Ki-67, age at diagnosis, or tumor area (Table 1). However, SPINK1-positive cases had significantly shorter progression-free survival compared with SPINK1-negative samples ($P = 0.0012$; Fig. 2B). Fifty-seven percent (12 of 21) of the SPINK1-positive and 31% (51 of 165) of the SPINK1-negative cases progressed during follow-up. According to multivariate analysis, SPINK1 expression was an independent prognostic marker, together with diagnostic PSA and Gleason score (Table 2).

The association between the presence of *TMPRSS2:ERG* fusion and SPINK1 expression was also examined. SPINK1 expression was seen in 12 of 110 (11%) *TMPRSS2:ERG* fusion-negative samples and in 7 of 60 (12%) *TMPRSS2:ERG* fusion-positive cases. There was no significant correlation between *TMPRSS2:ERG* fusion status and SPINK1 expression ($P = 1.000$; Table 3).

Discussion

The frequency of *TMPRSS2:ERG* fusion in the prostate needle biopsy specimens was 34%, which is about the same

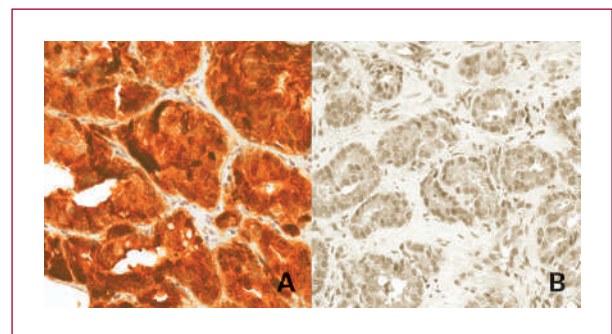


Fig. 3. An example of SPINK1-positive (A) and SPINK1-negative (B) staining in prostate cancer biopsy specimens.

Table 2. Multivariate analyses of the prognostic markers

Variable	RR (95% CI)	P
SPINK1*	2.3 (1.1-4.6)	0.038
PSA (>20 vs ≤20 ng/mL)	2.5 (1.4-4.3)	0.000
Gleason score (>7 vs 7 vs <7)	2.3 (1.4-3.0)	0.000
T stage (3+4 vs 1+2)	Nonsignificance	
Ki-67 staining (>10% vs >5%-10% vs ≤5%)	Nonsignificance	

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

*SPINK1 expression classified as positive when the intensity was 3 and the surface area stained was >10%.

as we have previously found in both prostatectomy-treated and castration-resistant prostate cancers (10). The *TMPRSS2:ERG* rearrangement was not associated with T stage, M stage, or diagnostic PSA. Although the frequency of the fusion seemed to be higher in high-Gleason score tumors, the association was not statistically significant. The findings are in concordance with our previous data in prostatectomy-treated patients (10). However, unlike in the prostatectomy-treated patients, we did find an association between the fusion and high proliferation rate as defined by Ki-67 staining. This may suggest that in more advanced tumors *TMPRSS2:ERG* fusion is affecting cell proliferation, whereas that is not the case in early cancer.

There was no association between *TMPRSS2:ERG* fusion and progression-free survival. Previous studies have analyzed the association of the fusion with prognosis in prostatectomy-treated or watchful waiting materials. The outcome in these studies has been variable. Some have indicated that the fusion is associated with poor prognosis (6, 16–18) and some with good prognosis (10, 19), whereas others have not found any association (9, 20, 21). Some studies have suggested that fusion due to deletion, not by translocation, as well as gain of the fusion gene is associated with aggressive phenotype of the disease (18, 21, 22). Here, we did not find any association between prognosis and different forms of the fusion. Although the follow-up

time was relatively long (median, 5 y) in our material, we were not able to study the association of the fusion with prostate cancer-specific survival. The number of prostate cancer deaths was too low. Thus, larger materials should be studied. However, because the progression-free time has been shown to predict survival, one may assume that the fusion is not associated with survival either. Recently, Boormans et al. (23) showed that the presence of *TMPRSS2:ERG* fusion transcript does not predict response to endocrine therapy in node-positive hormone-naïve prostate cancer. Our data confirm this finding in a larger patient cohort.

The frequency of SPINK1-positive cases was 11%, which is about the same as has previously been found in prostatectomized patients (13). SPINK1 expression was not associated with any of the clinicopathologic variables studied. However, the expression was strongly associated with short progression-free survival and, together with high pre-treatment PSA and Gleason score, SPINK1 expression was an independent marker of poor prognosis. To confirm the previously published (13) association of SPINK1 and prognosis in prostatectomy-treated patients, we also stained the prostatectomy specimens ($n = 180$) with the same anti-SPINK1 antibody. SPINK1 expression was not statistically significantly associated with prognosis, although patients with high SPINK1 expression had poorer progression-free survival (Supplementary Fig. S1). The difference was not statistically significant due to the low absolute number of SPINK1-positive cases. With a larger material, the difference would most likely have been significant. Because SPINK1 expression seems to be associated with poor prognosis in both prostatectomy-treated (13) and endocrine-treated patients, it seems to be a general biomarker of aggressive prostate cancer. Whether SPINK1 expression is truly associated with prostate cancer-specific survival remains to be confirmed.

The reason why SPINK1 is associated with aggressive phenotype of prostate cancer is unclear. Tomlins et al. (13) showed that SPINK1 expression is associated with invasive phenotype. On the other hand, it has been shown that SPINK1 promotes proliferation of pancreatic cancer cells through epidermal growth factor receptor (24). It has also been shown that the expression of epidermal growth factor receptor is associated with poor prognosis in endocrine-treated prostate cancer (25). The secretion

Table 3. Association between *TMPRSS2:ERG* fusion and SPINK1 expression

	SPINK1 expression– negative (%)	SPINK1 expression– positive (%)	Total (%)
Fusion-negative	98 (89)	12 (11)	110 (100)
Fusion-positive	53 (88)	7 (12)	60 (100)
Total	151	19	170

NOTE: $P = 1.000$; Fisher's exact test.

of SPINK1 is induced by interleukin 6 in hepatoblastoma cells (26), and interleukin 6 is produced by periprostatic adipose tissue of aggressive prostate cancer (27). Thus, it can be speculated that the stroma of aggressive prostate cancer may produce interleukin 6, which could induce the expression of SPINK1 in prostate cancer cells, leading to activation of the epidermal growth factor receptor signaling pathway. However, this hypothesis needs to be experimentally tested.

It has previously been suggested that SPINK1 is expressed only in *TMPRSS2:ERG* fusion-negative tumors (13). Here, we did not find such mutual exclusivity. In contrast, the frequency of the SPINK1 expression was the same in both fusion-positive and fusion-negative tumors. This was also the case in our prostatectomy material (Supplementary Table S1). We used the same antibody as Tomlins et al. (13), who found an association between the fusion and SPINK1 in prostatectomy material. However, we used slightly different criterion for SPINK1 positivity. We think the scoring of SPINK1 immunostaining with this antibody was straightforward. Also, the frequency of SPINK1 positivity was about the same as in the study by Tomlins et al. (13). One difference between our study and the study by Tomlins et al. (13) is the method of assessing the fusion. In the study by Tomlins et al. (13), the rearrangement was detected by two-color break-apart FISH assay, which identifies unspecified rearrangement in the region near *TMPRSS2* and *ERG*. We, on the other hand, used a three-color FISH assay that identifies a definite fusion between these two genes.

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In conclusion, the lack of association between the *TMPRSS2:ERG* fusion and prognosis in hormonally treated patients indicate that the fusion is not associated with androgen responsiveness of prostate cancer. Thus, the clinical variability in the response to endocrine therapy cannot be due to the *TMPRSS2:ERG* fusion. The association between progression-free survival and SPINK1 expression suggests that SPINK1 is also a prognostic biomarker in endocrine-treated prostate cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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