TMPRSS2:*ERG* Fusion Identifies a Subgroup of Prostate Cancers with a Favorable Prognosis

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Abstract

Purpose: Our aim was to assess the frequency of *ERG* overexpression and *TMPRSS2*:*ERG* rearrangement in prostate cancer and their association with clinicopathologic variables and outcome.

Experimental Design: The presence of the *TMPRSS2*:*ERG* rearrangement was studied by reverse transcription-PCR and fluorescence *in situ* hybridization in 19 prostate cancer xenografts and 7 prostate cancer cell lines. The expression of *ERG* was studied in the xenografts and cell lines and in 49 freshly frozen clinical prostate samples by quantitative reverse transcription-PCR. The frequency of the *TMPRSS2*:*ERG* fusion in clinical prostate cancer (n = 253) on tissue microarrays was assessed by three-color fluorescence *in situ* hybridization.

Results: Seven of 19 (37%) of the xenografts overexpressed *ERG* and had *TMPRSS2*:*ERG* rearrangement. Two xenografts, representing small cell carcinomas, also contained the fusion but did not express *ERG*. In clinical tumor specimens, the overexpression of *ERG* was associated with the rearrangement (P = 0.0019). Fifty of 150 (33%) of the prostatectomy specimens and 28 of 76 (37%) of the hormone-refractory prostate cancers on the tissue microarrays carried the *TMPRSS2*:*ERG* rearrangement. It was associated with longer progression-free survival in patients treated by prostatectomy (P = 0.019), and according to multivariate analysis, it was an independent predictor of favorable outcome (relative risk, 0.54; 95% confidence interval, 0.30-0.98). The fusion was not associated with Gleason score, pT stage, diagnostic prostate-specific antigen, or cell proliferation activity in prostatectomy specimens nor with the *AR* gene amplification in hormone-refractory tumors.

Conclusions: The *TMPRSS2*:*ERG* rearrangement can be found in about one third of prostate cancers. A subgroup of prostate cancer patients with a good prognosis may be identified by the rearrangement.

Recent studies suggest that >50% of prostate tumors may carry a chromosomal rearrangement on chromosome 21q22 (1–4). The genes involved are the androgen-inducible *TMPRSS2* (transmembrane protease, serine 2) and an ETS family transcription factor, *ERG* [v-ets erythroblastosis virus E26 oncogene like (avian)]. As a result of the rearrangement, the expression of *ERG* becomes androgen regulated.

TMPRSS2 is highly expressed in normal and neoplastic prostate in an androgen-dependent manner (5, 6). Its significance is unknown, as knockout mice show a normal phenotype, indicating that the gene is redundant (7). *ERG* is a transcription factor often involved in oncogenic translocations in Ewing's sarcoma and myeloid leukemias (8). It has been shown to interact with a histone H3-specific methyltransferase (ESET) and may hence participate in the epigenetic silencing of downstream target genes (9).

The genomic breakpoints and the fusion transcripts of *TMPRSS2*:*ERG* are not uniform (1–3, 10–14). To date, ~20 different variants of *TMPRSS2*:*ERG* have been described. The two most common variants (*TMPRSS2* exon1:*ERG* exon4/5) presumably produce a full-length, functional ERG and have also been seen together, implicating that differential splicing of the transcript adds to the diversity (12, 14, 15). The distance between the two genes is a mere 2.8 Mb, and they lie in the same orientation, facilitating the creation of a functional fusion. Indeed, in most cases, the rearrangement seems to simply involve an interstitial deletion between the genes (3, 10, 15, 16).

The reported frequencies for the *TMPRSS2*:*ERG* rearrangement vary from 15% to 78%, depending on sample sets and detection method. Some controversy also remains about the

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association of the fusion with clinical variables, such as Gleason score and pT stage, as well as prognosis. Therefore, we sought to determine the frequency of the *TMPRSS2:ERG* fusion in prostate cancer as well as its association with clinicopathologic variables and outcome.

Materials and Methods

Cell lines and xenografts. Four prostate cancer cell lines (LNCaP, DU-145, PC-3, and 22Rv1) were obtained from the American Type Culture Collection and grown under recommended conditions. The LAPC-4 prostate cancer cell line was kindly provided by Dr. Charles Sawyers (Memorial Sloan-Kettering Cancer Center, New York, NY), and the VCaP and DuCaP prostate cancer cell lines were provided by Dr. Jack Schalken (Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands). The LAPC-4 cells were maintained in 1× Iscove's medium with 15% fetal bovine serum and 1% penicillin-streptomycin/L-glutamine. The VCaP and DuCaP were maintained in the same medium as LNCaP. Nineteen human prostate cancer xenografts of the LuCaP series were made available by one of the authors (R.L.V.) and have been described elsewhere (17, 18).

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

Forty-nine freshly frozen clinical prostate samples, including 9 benign prostatic hyperplasia, 28 untreated primary prostate carcinomas, and 12 locally recurrent hormone-refractory prostate carcinomas, were obtained from the Tampere University Hospital (Tampere, Finland). The samples were confirmed to contain a minimum of 60% cancerous or hyperplastic cells by H&E staining. The benign prostatic hyperplasia samples were obtained from prostatectomies of prostate cancer patients but controlled not to contain any cancer cells. The hormone-refractory prostate cancer samples were from patients undergoing transurethral resection of the prostate for urethral obstruction despite ongoing androgen ablation therapy. The median time from the onset of hormonal treatment to progression was 38 mo (range, 15-68).

Two hundred and forty-eight formalin-fixed, paraffin-embedded prostate cancer samples from prostatectomies, and 127 formalin-fixed, paraffin-embedded locally recurrent hormone-refractory prostate ancers from transurethral resection of the prostate, were obtained from Tampere University Hospital. The prostatectomy specimens were regraded for Gleason score by a pathologist. According to standard practice, the serum prostate-specific antigen (PSA) levels of prostatectomy patients were monitored at Tampere University Hospital for 1 y after the operation and subsequently at least once a year at the local health centers of the Hospital District. Thus, serum PSA values and dates were retrieved, in addition to the patient files at Tampere University Hospital, also from health centers. The median follow-up time was 66 mo (range, 3.4-219 mo). Detectable PSA values (≥0.5 ng/mL) in two consecutive measurements or the emergence of metastases was considered as a sign of progression. For the hormone-refractory samples, the median time from diagnosis (onset of treatment) to transurethral resection of the prostate (progression) was 24 mo (range, 1-144 mo).

Representative regions of the formalin-fixed, paraffin-embedded tissue blocks were chosen for tissue microarray (TMA) construction. The TMAs were constructed with a manual tissue arrayer (Beecher Instruments) and a 1-mm needle according to the manufacturer's instructions. H&E staining of adjacent sections was used to ascertain that the tissue cores contained a sufficient amount of cancerous regions for analysis. Fifty-four (14%) samples were lost during processing of the TMA blocks and 68 (18%) tissue cores were determined noncancerous with the H&E staining. Two hundred and fifty-three representative samples, including 171 untreated prostate cancer specimens and 82 hormone-refractory prostate cancers, were finally suitable/available for analysis.

To evaluate the proliferation activity of the tumors, the TMAs were immunostained with an antibody against Ki-67 (1:1,500 dilution, MM1; Novocastra Laboratories Ltd.) using PowerVision+ Poly-HRP Histostaining kit (ImmunoVision Technologies Co.), as described elsewhere in detail (19).

RNA extraction and cDNA synthesis. Total RNA was extracted from the cell lines with the Trizol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. SuperScript II reverse transcriptase (Invitrogen) and oligo(dT)$_{12\text{-}18}$ primers were used according to the enzyme manufacturer's instructions to synthesize first-strand cDNA with 2 μg of total RNA as template. Another lot of cDNAs was synthesized with random hexamer primers.

Total RNA was isolated from freshly frozen pieces of xenograft tissue with the Tri-Pure reagent and protocol (Roche Diagnostics), and cDNAs were synthesized using oligo(dT)$_{12\text{-}18}$ primers and 2 μg of total RNA, as above. Another lot of total RNAs was isolated with the Qiagen RNeasy Mini kit (Qiagen, Inc.), and first-strand cDNAs were synthesized using random hexamer primers and 2 μg of total RNA as template. The RNA extraction from the clinical samples and cDNA synthesis using oligo(dT)$_{12\text{-}18}$ primers is described in detail by Linja et al. (20).

Reverse transcription-PCR. The presence of the *TMPRSS2:ERG* fusion transcript was initially assayed in the cell lines and xenografts by reverse transcription-PCR (RT-PCR) with fusion-specific primers (*TMPRSS2* RT forward, 5′-caggaggcggaggcgga-3′; *TMPRSS2:ERG* RT reverse, 5′-ggcgttgtagctgggggtgag-3′), essentially as previously described (11). Briefly, the amplification reactions were done using the random hexamer-primed cDNA as template and AmpliTaq DNA Polymerase LD enzyme (Perkin-Elmer). Standard three-step PCR with annealing temperature of 65°C was done, after which the samples were run in 1.5% agarose and stained with ethidium bromide. *ERG* expression in the clinical samples was measured from the oligo(dT)-primed cDNAs by real-time quantitative RT-PCR (Q-RT-PCR) with the LightCycler apparatus and the LightCycler FastStart DNA SYBR Green I kit (Roche Diagnostics), essentially as described (21). The used primers (*ERG* forward, 5′-tcttggaccaacaagtagcc-3′; *ERG* reverse, 5′-gtcgggatccgtcatcttg-3′) recognize both known isoforms at the 3′-end of the mRNA and should also recognize all forms of rearranged *ERG*. The *ERG* expression levels were normalized to the expression levels of TATA box-binding protein as described (21). To ensure that only the specific PCR product was amplified, a melting curve analysis, as well as a 1.5% agarose gel electrophoresis, was done.

Fluorescence* in situ *hybridization. Three-color fluorescence *in situ* hybridization (FISH) was carried out on interphase spreads of the cell lines, on 5-μm frozen sections of the xenografts, and on 6-μm sections of the TMAs, essentially according to Saramäki et al. (18). Locus-specific bacterial artificial chromosome probes for *ERG* (RP11-164E1), *TMPRSS2* (RP11-814F13), and the region between the two (RP11-367P1) were labeled by nick translation with digoxigenin-dUTP, Alexa Fluor 594-dUTP (Molecular Probes), and biotin-dUTP, respectively. After washing and staining with anti-digoxigenin-FITC and streptavidin-Pacific Blue (Vector Laboratories), the sections were embedded in Vectashield antifade solution (Vector Laboratories) containing 0.001 mol/L 4′,6-diamidino-2-phenylindole as counterstain. The FISH signals were scored with an Olympus BX5 epifluorescence microscope equipped with a charge-coupled device camera. To examine all colors and signals at once in thick tissue sections, stacks of nine images were captured with the Image-Pro Plus 6.1 software (Media Cybernetics, Inc.) through each filter set and combined to produce an RGB image with an extended depth of focus.

For *androgen receptor* (*AR*) gene copy number analysis previously described (18), dual-color FISH protocols and a digoxigenin-dUTP–labeled locus-specific P1 probe for *AR* (LCG-P1AR) and a Texas red–labeled or FITC-labeled pericentromeric alphoid repeat probe for chromosome X (DXZ1) were used.

Statistical analyses. Fisher's exact, χ^2, Mann-Whitney *U*, and Student's *t* tests were used to determine the association between the *TMPRSS2:ERG* rearrangement and the clinicopathologic variables.

Kaplan-Meier survival analysis and Cox regression hazard model were used to evaluate the prognostic value of the rearrangement.

Results

RT-PCR detected the *TMPRSS2*:*ERG* fusion transcript in only VCaP and DuCaP of the cell lines (LNCaP, DU-145, PC-3, LAPC-4, 22Rv1, VCaP, and DuCaP) studied, as expected based on previously published data (1). Three-color FISH on the VCaP and DuCaP cell lines further confirmed the presence of the fusion (Fig. 1A). They also overexpressed *ERG* according to Q-RT-PCR.

Seven of 19 of the xenografts contained the *TMPRSS2*:*ERG* fusion transcript and showed fused signals by FISH. They also overexpressed *ERG*, as determined by the Q-RT-PCR (Fig. 2). Two xenografts (LuCaP49 and LuCaP93) contained the rearrangement according to FISH analysis but did not express *ERG* or contain the fusion transcript (Fig. 1B). Seventeen of 28 (61%) untreated clinical prostate carcinomas and 6 of 12 (50%) hormone-refractory clinical tumors overexpressed *ERG*. None of the benign prostatic hyperplasia samples showed elevated *ERG* expression. In the samples (n = 16) where the *TMPRSS2*:*ERG* rearrangement could be evaluated by FISH, the overexpression was associated with it (P = 0.0019; Fig. 3).

TMPRSS2:*ERG* fusion was evaluable by FISH in 226 of 253 (89%) of the representative samples on the TMAs. Fifty of 150 (33%) prostate cancers from prostatectomies and 28 of 76 (37%) hormone-refractory cancers contained the *TMPRSS2*:*ERG* rearrangement. An example of a specimen carrying the fusion is shown in Fig. 1C. In the prostatectomy specimens, the fusion was not associated with Gleason score, pT stage, diagnostic PSA, or Ki-67 immunostaining (Table 1). However, according to Kaplan-Meier analysis, it was significantly associated with longer progression-free survival (P = 0.019, Mantel-Cox test; Fig. 4). Multivariate analysis was used to evaluate the independence of the rearrangement as a prognostic factor. The other variables that were included in the multivariate analysis, pT stage (3 versus 2), Gleason score (>7 versus 7 versus <7), diagnostic PSA value (30 versus 10-29.9 versus 0-9.9 ng/mL), and Ki-67 immunostaining (16% versus 2-15% versus 0-1%), have all shown prognostic value in univariate analyses (19). The *TMPRSS2*:*ERG* fusion showed independent prognostic value with relative risk of 0.54 and 95% confidence interval of 0.30 to 0.98, as did pT stage (relative risk, 2.2; 95% confidence interval, 1.29-3.67) and Ki-67 staining (relative risk, 3.11; 95% confidence interval, 0.97-9.97).

Of the cell lines, VCaP and DuCaP were found to contain an amplification of *AR* gene by FISH (Fig. 1D). The *AR* gene amplification was evaluable in 61 (74%) of the hormone-refractory prostate cancers on the TMAs. There was no association between *AR* amplification and the *TMPRSS2*:*ERG* fusion (P = 1.000; Table 2).

Discussion

The frequency of the *TMPRSS2*:*ERG* rearrangement in untreated cancer samples from prostatectomies (n = 150) was 33% according to three-color FISH analysis. It has previously been suggested that the frequency could be 40% to 70%. In most studies, the sample sets have, however, been smaller and the detection method has been RT-PCR (1, 2, 4, 11, 12). In the studies where large sample sets have been used, the method of choice has usually been the two-color break-apart FISH assay, which, rather than identifying a definite fusion of *TMPRSS2* and *ERG*, only shows an unspecified rearrangement in the region. This may lead to overestimation of the fusion frequency. Rajput et al. (22) have recently used a three-color FISH assay similar to ours and detected the *TMPRSS2*:*ERG*

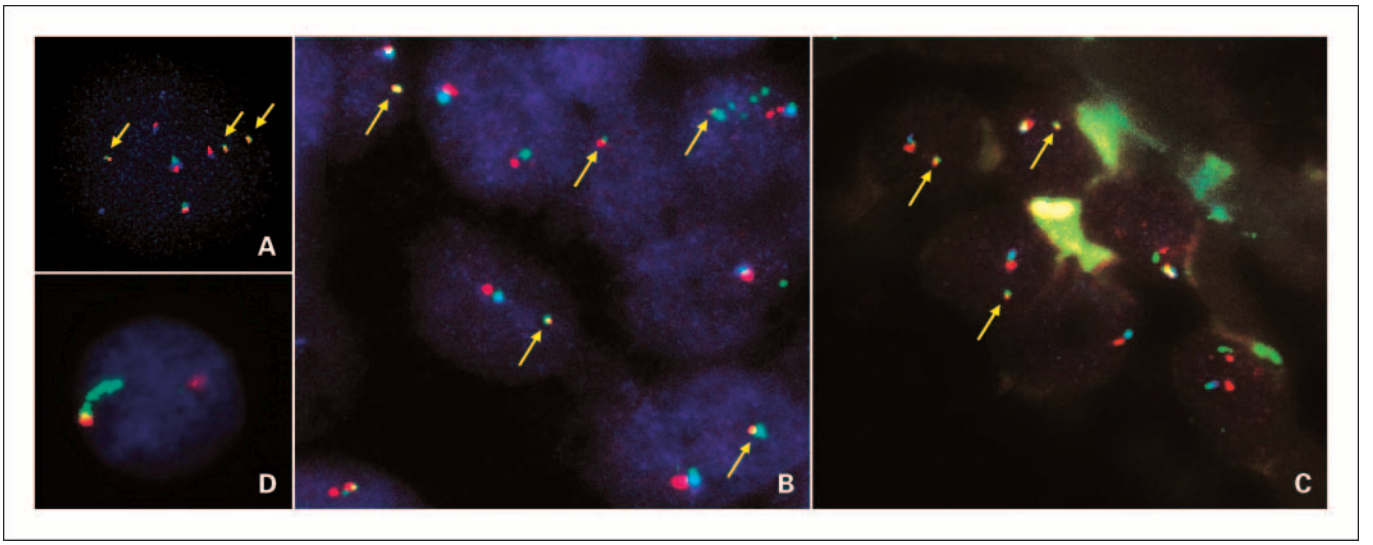

Fig. 1. Three-color FISH analysis of *TMPRSS2*:*ERG* fusion. The signals are red for *TMPRSS2* (RP11-814F13), green for *ERG* (RP11-164E1), and blue for RP11-367P1. The blue signal normally located between the red and green is lost in abnormal chromosomes. *A,* VCaP prostate cancer cell line showing seven copies of *TMPRSS2* (*red*) and five copies of *ERG* (*green*). Arrows, three pairs of *TMPRSS2* and *ERG* are seen as a combined red + green = yellow signal. Two apparently normal chromosome 21s, as well as two pairs of red and blue signals, are also seen. *B,* arrows, LuCaP93 showing fusion of *TMPRSS2* and *ERG*. *C,* a prostate cancer specimen showing *TMPRSS2*:*ERG* rearrangement as a yellowish signal (*arrows*) and a normal chromosome 21q22 locus (*red, blue*, and *green*). The proximity of the normal signals in some nuclei may result in white (all three), cyan (*ERG* + RP11-367P1), or magenta (*TMPRSS2* + RP11-367P1) signals. *D,* amplification of *AR* gene (*green*) in VCaP. Red, the amplification presents as a large homogenously staining region near chromosome X centromere signal. A normal pair of signals is also seen.

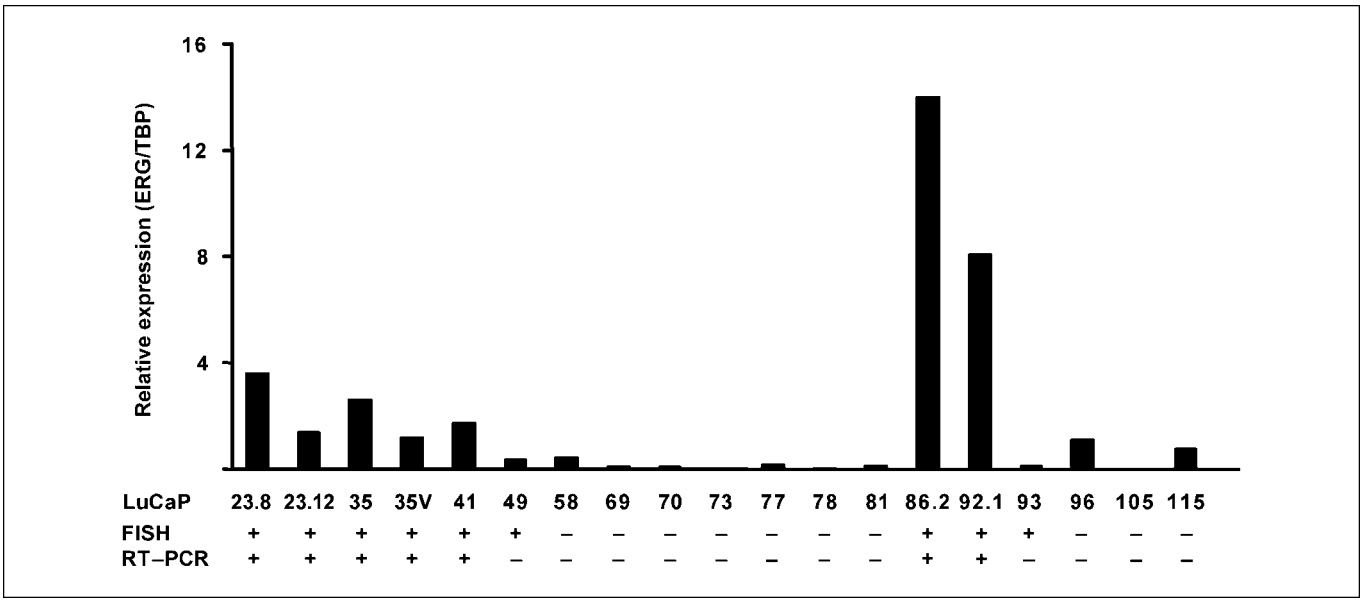

Fig. 2. Relative expression of *ERG* in the xenografts by real-time Q-RT-PCR. The *TMPRSS2*:*ERG* fusion status according to FISH and RT-PCR is shown below. The two samples with discrepant results are from the xenografts established from the AR-negative small cell carcinomas of prostate. *TBP*, TATA box-binding protein.

fusion in 36 of 101 (36%) of prostatectomy-derived prostate cancer samples. They also used the break-apart assay and found that five cases that were positive according to the break-apart assay were fusion negative by the three-color *TMPRSS2*:*ERG* fusion assay. The three-color FISH assay used here seemed to be specific and sensitive because all fusion-positive samples, for which also cDNA was available, overexpressed *ERG* by Q-RT-PCR, and all but one fusion-negative case showed only low expression of the gene.

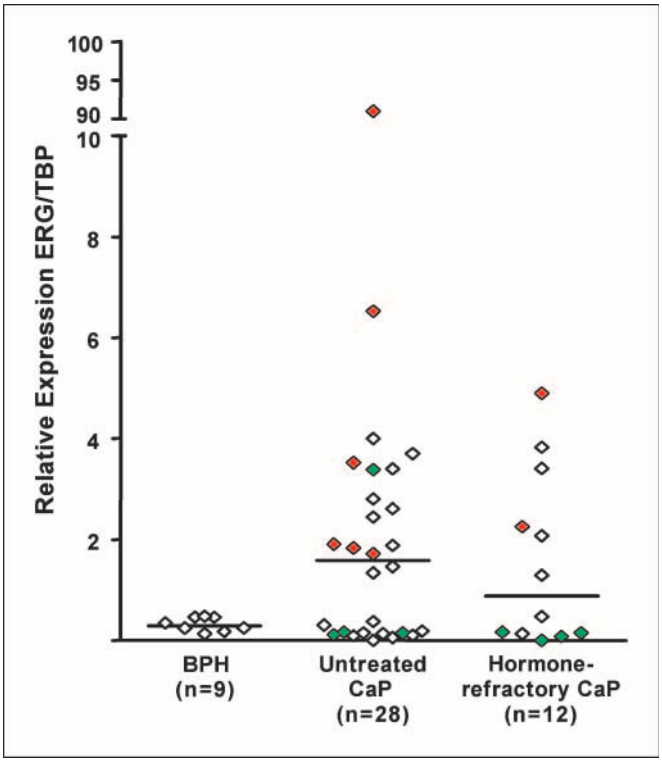

Fig. 3. Relative expression of *ERG* in the clinical prostate samples by Q-RT-PCR. Red, cases with *TMPRSS2*:*ERG* fusion according to FISH; green, fusion-negative cases; white, cases without information of the fusion. BPH, benign prostatic hyperplasia; CaP, prostate cancer.

In addition to the methodologic differences, the higher frequency of the rearrangement in many studies may be to be due to differences in the sample materials. Studies using RT-PCR, as a detection method, have consistently found a higher fraction of prostate tumors with the fusion (2, 4, 11). RT-PCR requires freshly frozen tumor samples, which also need to be highly representative, especially if microdissection is not used. Thus, these materials do usually not represent unselected consecutive cases. For example, our Q-RT-PCR data of the untreated clinical prostate cancers, derived also from prostatectomies (n = 28), indicated overexpression (i.e., *TMPRSS2*:*ERG* fusion) in ~60% of the cases. This material is highly selected due to the above-mentioned reasons. The watchful waiting cohort of Demichelis et al. (23) represented an unselected material of very early, largely nonprogressive cancers, and there, the frequency was even lower, 15%, indicating once again the significance of the sample material.

In the hormone-refractory samples, the frequency of the *TMPRSS2*:*ERG* fusion was about the same (37%) as in the untreated prostate cancers (33%). Thus, it seems that there is no selection for or against the rearrangement during disease progression. Neither was the fusion associated with the progression-free time during the hormonal therapy (data not shown), suggesting that the rearrangement is not connected to hormone responsiveness of prostate cancer.

Contradicting results have been published about the prognostic value of the *TMPRSS2*:*ERG* rearrangement. Some studies have found an association between the rearrangement and poor prognosis, whereas others have shown *ERG* overexpression or the rearrangement to be associated with good prognosis (11, 23–27). In our material, the fusion was significantly associated with good prognosis, although not with Gleason score, pT stage, diagnostic PSA, or proliferation index, all

Table 1. Association of clinicopathologic variables with the *TMPRSS2*:*ERG* fusion

Variable	*TMPRSS2:ERG* fusion according to FISH		*P*
	No fusion	Fusion	
Prostatectomy specimen, *n* (%)	100 (67)	50 (33)	
Hormone-refractory tumors, *n* (%)	48 (63)	28 (37)	0.658*
Prostatectomy specimens			
Gleason score, *n* (%)			
<7	35 (63)	21 (37)	
7	53 (70)	22 (30)	
>7	10 (63)	6 (37)	0.576*
pT stage, *n* (%)			
pT2	66 (67)	33 (33)	
pT3	34 (67)	17 (33)	1.000*
PSA (mean ± SD)	17.15 ± 13.97	11.78 ± 6.56	0.100 †
Ki-67 (mean ± SD)	7.19 ± 7.38	6.43 ± 4.65	0.773 †
Age (mean ± SD)	62.3 ± 5.10	64.0 ± 4.54	0.045 ‡

*χ^2 test.
†Mann-Whitney *U* test.
‡Student's *t* test.

well-known prognostic markers. Thus, in the multivariate analysis, the *TMPRSS2*:*ERG* rearrangement was actually an independent prognostic marker. Our data are consistent with that of Petrovics et al. (25), who showed an association between high expression of *ERG*, measured by Q-RT-PCR, and longer PSA recurrence-free survival in patients treated by prostatectomy. In addition, Winnes et al. (26), who evaluated the *TMPRSS2*:*ERG* fusion by RT-PCR in a set of 50 needle biopsies of palpable prostate tumors, reported a trend toward longer PSA progression-free survival in fusion-positive cases. In contrast, a positive association between the rearrangement, determined by break-apart FISH assay, and prostate cancer–specific death has been shown in watchful waiting cohorts (23, 24). The material of Demichelis et al. (23), consisted of T1a-b tumors, representing mainly transitional zone carcinomas, with low likelihood of recurrence, and Attard et al. (24) examined conservatively managed prostate cancers, most of which had low Gleason score and/or clinical stage. Thus, the discrepancies are most likely due to the differences in materials, and therefore, the results are not comparable. The other published prognostic studies are clearly smaller in the size of the material (11, 27).

The two xenografts that contained the rearrangement according to FISH analysis, but did not express *ERG* (LuCaP49 and LuCaP93), have both been established from small cell carcinomas of the prostate, and they are AR negative (21). In addition, Hermans et al. (15) have reported four androgen-independent xenografts (PC series) that contain the rearrangement, detected by FISH, which do not express *ERG*. This implies that, in some cases, the *ERG* (over)expression brought on by the *TMPRSS2*:*ERG* fusion no longer contributes to the progression of the disease. The fact that the xenograft samples derived from small cell carcinomas contained the fusion indicates that also this rare type of aggressive prostate cancer arises originally from androgen-dependent cells.

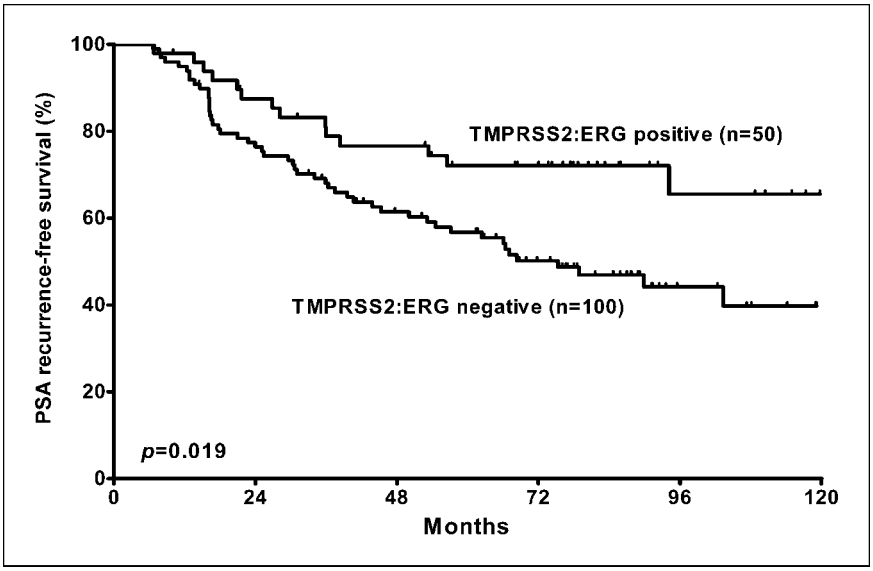

Fig. 4. Kaplan-Meier analysis of the progression-free survival of prostatectomy-treated patients. *TMPRSS2*:*ERG* fusion-positive patients had a statistically significantly longer progression-free survival ($P = 0.019$) than the fusion-negative patients.

Table 2. Association of AR gene amplification and *TMPRSS2*:*ERG* fusion

	AR amplification negative, *n* (%)	AR amplification positive, *n* (%)	Total, *n* (%)
Fusion negative	31 (51)	8 (13)	39 (64)
Fusion positive	17 (28)	5 (8)	22 (36)
Total	48 (79)	13 (21)	61 (100)

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

We used *ERG* primers that recognize the 3′-end of the transcript for the quantitative expression analysis of *ERG*. Although the transcripts amplified were not definitively fusion derived, previous studies have shown that *ERG* overexpression is practically always due to a genomic rearrangement with *TMPRSS2* (1, 3). This was also shown by the correlation between the *ERG* expression and *TMPRSS2*:*ERG* rearrangement in our limited set of clinical samples ($n = 16$) for which both analyses were possible as well as in the xenografts (AR-negative xenografts excluded) and cell lines. Therefore, *ERG* (over)-expression, in general, may be considered an indication of the *TMPRSS2*:*ERG* fusion.

Because the expression of *ERG* becomes androgen regulated in *TMPRSS2*:*ERG* fusion-positive prostate cancer and the VCaP cell line, which contains the fusion, carries also *AR* gene amplification, we hypothesized that the two aberrations might be associated with each other. However, although the frequencies of the aberrations are similar, there was no association between them, and thus, the events seem to be independent of each other.

The *TMPRSS2*:*ERG* fusion was assessed in an unselected sample set of prostatectomy-treated prostate carcinoma, and our data show that the frequency of *TMPRSS2*:*ERG* fusion is ~30%. In hormone-refractory prostate cancer, the frequency seems to remain the same, suggesting that no selection for or against the rearrangement takes place during disease progression. The fusion seems to identify a distinct subgroup of tumors, which by many variables, such as Gleason score, does not differ from fusion-negative tumors but has a favorable prognosis.

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