Mapping of *TMPRSS2-ERG* fusions in the context of multi-focal prostate cancer

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***TMPRSS2-ERG* gene fusion leading to the androgenic induction of the *ERG* proto-oncogene expression is a highly prevalent oncogenic alteration in prostate tumor cells. Prostate cancer is a multi-focal disease, and the origins as well as biological contribution of multiple cancer foci remain unclear with respect to prostate cancer onset or progression. To assess the role of *TMPRSS2-ERG* alteration in prostate cancer onset and/or progression, we have evaluated the status of fusion transcripts in benign glands, prostatic intraepithelial neoplasia (PIN) and multiple cancer foci of each prostate. Quantitative expression of *TMPRSS2-ERG* fusion type A and C transcripts was analyzed in benign, tumor and PIN areas, selected from whole-mount radical prostatectomy slides. *TMPRSS2-ERG* expression was correlated with clinicopathological features. Overall, 30 of 45 (67%) patients exhibited *TMPRSS2-ERG* fusion transcripts in at least one tumor focus. Of 80 tumor foci analyzed, 39 had *TMPRSS2-ERG* fusion (type A only: 30, type C only: 2, both types A and C: 7), with predominant detection of the *TMPRSS2-ERG* fusion type A (27/30, 90%) in the index tumors. Of 14 PIN lesions, 2 were positive for type A fusion. Frequent presence of the *TMPRSS2-ERG* in index tumors suggests critical roles of *ERG* alterations in the onset and progression of a large subset of prostate cancer. However, heterogeneity of the *TMPRSS2-ERG* detection in the context of multiple cancer foci and its frequency in PIN also support the role of other genomic alterations in the origins of prostate cancer.**

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Prostate cancer has been recognized as a multi-focal disease.^{1–3}

Currently, in our series of over 1150 radical prostatectomies most specimens contain two or more independent tumors. It is still unclear whether each of these cancer foci contributes to patient's prognosis after the radical prostatectomy. We and others found that the larger tumors typically contain poorly differentiated foci.^{1,2} Stamey *et al*⁴ reported that percent Gleason grade 4/5, cancer volume of the largest tumor, positive lymph node findings and intraprostatic vascular invasion have been independently associated with prostate cancer progression.

Intertumoral as well as intratumoral heterogeneity with respect to genomic alterations has also been noted in multiple foci.⁵ The biological significance of multiple independent foci of prostate cancer, histological grade heterogeneity within each tumor focus and difference in the anatomical location of the tumor are still unclear.

Recent reports of prevalent chromosomal rearrangements/translocations, leading to the activation of *ETS* transcription factors (predominantly *ERG*) through androgen receptor-inducible *TMPRSS2* gene promoter,⁶ underscore the critical role of *ERG* overexpression in prostate cancer.⁷ Subsequent reports,^{8–12} including our study,¹³ have shown high frequency of *TMPRSS2-ERG* fusion and/or overexpression in clinically localized prostate cancers.

TMPRSS2 encodes an androgen-dependent transmembrane serine protease, which is strongly expressed in both normal tissue and prostate cancer. *ETS* transcription factors regulate the expression of genes with important cancer-relevant biological

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processes, such as cell growth, differentiation, and transformation.¹⁴ *TMPRSS2-ERG* fusion may lead to prostate cancer cell-associated androgenic regulation of *ERG*⁶ in prostate cancer cells. However, the biologic role of *ERG* overexpression in prostate cancer development and progression remains to be defined. Since *TMPRSS2-ERG* fusions lead to frequent alterations (>60%) of the *ERG* proto-oncogene in early-stage prostate cancer, their evaluation in preneoplastic cells as well as multi-focal lesions from the same patient has potential to define its role in the prostate cancer onset, progression, and heterogeneity. This study analyzes *TMPRSS2-ERG* alterations in prostatic intraepithelial neoplasia (PIN) and multi-focal cancers sampled from whole-mount prostate glands of prostate cancer patients.

Materials and methods

Cell Culture

Human prostate cancer cell line VCaP (cat. no. CRL-2876; American Type Culture Collection, Rockville, MD, USA) with known *TMPRSS2-ERG* fusion was grown in Dulbecco's modified Eagle's medium containing 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 10% fetal bovine serum. VCaP cells were used as positive control for *TMPRSS2-ERG* fusion expression and were also used for determining the sensitivity of detecting *TMPRSS2-ERG* fusion.

TMPRSS2-ERG Fusion Measurements in Formalin-Fixed Paraffin-Embedded VCaP Cells

To develop a set of positive controls for *TMPRSS2-ERG* fusion assessment in tissue sections, VCaP cell pellets were fixed with formalin and processed as paraffin blocks. A paraffin block containing VCaP cells was sectioned and utilized for checking the sensitivity and reproducibility of *TMPRSS2-ERG* fusion in formalin-fixed paraffin-embedded (FFPE) material. We determined that a minimum of 100 pg of VCaP RNA was needed for a reproducible real-time RT-PCR-based *TMPRSS2-ERG* fusion detection assay. PCR primers and PCR conditions are given in the Quantitative real-time RT-PCR section below.

Whole-Mount FFPE-Prostate Specimens and Correlation of *TMPRSS2-ERG* Expression with Clinicopathological Data

This study was performed under IRB-approved protocols (WU no. 04-28030, and WU no. 04-2871-98k). Prostatectomy specimens from patients treated at Walter Reed Army Medical Center were sectioned and embedded as whole mounts at the Armed Forces Institute of Pathology. After surgery, radical prostatectomy specimens were immediately fixed in 10% buffered formalin and embedded as whole

mounts in paraffin. Each prostate was sectioned at 0.22 cm intervals in a transverse plane perpendicular to the long axis of the posterior surface of the prostate and completely embedded as whole mounts. The volume of each tumor was calculated in three dimensions (apex to base, right to left, and anterior to posterior) using the largest dimension in each direction. The volume did not include the shrinkage factor to correct for fixation and embedding. The tumor volumes of the measurable foci, from the largest (T1) to the smallest (T4) were recorded as T1–T4. The tumor dimension was not corrected for the actual tumor shape of the cross-section and, therefore, does not represent the true size of the tumor in cross-sectional area.

One 4- μ m whole-mount slide was stained with hematoxylin and eosin, and used for a control. The adjacent two 6- μ m sections were used for tissue dissection. Quantitative expression of the *TMPRSS2-ERG* was analyzed for a relationship with patient age at the time of surgery, race, clinical stage, preoperative PSA, prostatectomy pathology data (specimen size, tumor volume, grade, stage, and surgical margin status), and follow-up PSA data.

Definition of Non-Malignant Tissue and PIN

Epithelium was classified as histologically non-malignant when glands contained two cell layers: the luminal cells with inconspicuous nucleoli and basal cells. Native glands were classified as PIN, when the secretory cells showed nuclear anaplasia and basal cells were detectable.¹⁵ PIN adjacent to carcinoma (PIN located within 2 mm distance from the edge of tumor foci) and PIN distant from carcinoma (PIN located more than 2 mm distant from edge of tumor foci) were also evaluated for *TMPRSS2-ERG* fusion.

Tissue Micro-Dissections

Unstained 6- μ m-thick tissue sections on glass slides were deparaffinized once with citrasolve buffer (cat. no. 22-143-975; Fisher Scientific, Pittsburgh, PA, USA) for 10 min, rinsed twice with 100% ethanol for 10 min, and air-dried. Specific histological fields of interest were selected (and ink marked) under the microscope by a genitourinary pathologist. Micro-dissection was performed using a pipette with an ultrafine pointed tip (cat. no. 2149p; Molecular BioProducts, San Diego, CA, USA). The histological area of interest on the tissue section was gently scraped with the pipette tip and RNA extraction buffer (total 250 μ l RNA lysis buffer; 20 mM Tris (pH 7.4), 20 mM EDTA, 1% SDS, and 1 mg/ml proteinase K) until the selected cells became detached from the tissue section. Samples were transferred to Eppendorf tube and incubated overnight in 55°C water bath. After incubation, 750 μ l TRIzol (cat. no. 15596-026; Invitrogen, Carlsbad, CA, USA) was added into

the lysis buffer to extract RNA according to the manufacturer's recommendations. RNA was treated by DNase I (cat. no. 18068; Invitrogen) for 15 min at room temperature. RNA concentration was measured by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Quantitative Real-Time RT-PCR

Total RNA (100 ng) was reverse transcribed to cDNA using Omniscript reverse transcriptase (cat. no. 20513; Qiagen Technology, Germantown, MD, USA) and random hexamer primers in a volume of 20 μ l. For each PCR, cDNA equivalent to 22.5 ng of total RNA was used in a final volume of 30 μ l. Quantitative real-time PCR was performed using SYBR green master mix (cat. no. 4309155; Applied Biosystems, Foster City, CA, USA) in ABI PRISM 7700 sequence analyzer (Applied Biosystems). Each patient sample was analyzed in duplicate. Thermal cycling conditions were as follows: initial one-step incubation at 50°C for 2 min, followed by a single denaturation step at 95°C for 10 min. Two-step PCR was then carried out for 45 cycles with 95°C denaturation for 15 s, followed by annealing/extension at 60°C for 60 s. Fluorescence intensity measurements were made at the end of each extension phase. The following PCR primers were used in the study: *TMPRSS2-ERG* fusion A sense primer: CGCGGCAGGAAGCCTTAT (located in *TMPRSS2* exon 1), antisense primer: CGCGGTCATCTCTGTC TAGC (*ERG* exon 4); *TMPRSS2-ERG* fusion C sense primer: AGCGCGGCAGGAAGTCT (*TMPRSS2* exon 1), antisense primer: CCATGTAGCTGCCGTAGTT CATC (*ERG* exon 5); *PSA* sense primer: CCCACTG CATCAGGAACAAA, antisense primer: GAGCGGGT GTGGGAAGCT; β -2-microglobulin sense primer: TG ACTTTGTCACAGCCCAAGATA, antisense primer: ATTCCAAATGCGGCATATTC. A 9 pmol portion of each primer was used in the PCRs. RT-PCR for *PSA* and β -2-microglobulin served as RNA quality control. Each PCR product was independently confirmed for expected size by electrophoresis on the TBE-agarose gel (1.5%). Schematic diagrams of *TMPRSS2-ERG* fusion variants (types A and C) and sensitivity assay of quantitative RT-PCR for *TMPRSS2-ERG* fusion A using RNA from FFPE VCaP cells are shown in Figure 1.

Statistical Analysis

Clinicopathological parameters, including age at time of surgery, preoperative PSA level, race, clinical stage, pathological stage, Gleason score, surgical margin status, tumor volume and PSA recurrence were compared with the presence of *TMPRSS2-ERG* fusion. For this analysis, Student's *t*-test and Fisher exact test were used. To determine whether the *TMPRSS2-ERG* fusion is a prognostic factor for cancer progression, we performed survival

analysis, using PSA recurrence as primary end point. This was defined as two consecutive post-operative PSA ≥ 0.2 ng/ml. PSA recurrence-free survival curves were compared between patients with and without expression of *TMPRSS2-ERG* fusion using Kaplan–Meier unadjusted survival analysis method. Univariate Cox proportional hazard analysis was carried out to examine odds of PSA recurrence over time for all clinicopathological parameters, as well as *TMPRSS2-ERG* fusion. Hazard ratio and corresponding confidence interval and *P*-values are reported. *P*-value < 0.05 was adopted as statistically significant. The SAS version 9.1 was used for all data analysis.

Results

The detailed topographical data of *TMPRSS2-ERG* fusion in multiple tumor foci of each patient are summarized in Table 1. Representative whole-mount prostate sections of six patients with *TMPRSS2-ERG* fusion transcript status are also shown in Figure 2. Overall, 30 of 45 patients (67%) exhibited *TMPRSS2-ERG* fusion transcripts in at least one tumor focus. Heterogeneity of fusion transcripts in same patient specimen were apparent (Figure 2). Of 80 tumor foci analyzed, 39 (49%) had *TMPRSS2-ERG* fusion. In these 39 fusion-positive tumor foci, 30 demonstrated the *TMPRSS2-ERG* fusion type A only, 2 showed *TMPRSS2-ERG* fusion type C only, and 7 had both types A and C.

In PIN lesions, overall 2 of 14 foci were positive for fusion type A. In 5 of 14 cases, PIN was located adjacent to tumor (PIN located within 2 mm distance from edge of tumor foci) and 1 of these 5 foci was positive for *TMPRSS2-ERG* fusion type A. Among the PIN lesions distantly located (PIN located more than 2 mm distant from edge of tumor foci), 1 of 9 foci had *TMPRSS2-ERG* fusion type A.

Intriguingly, 2 of 45 RNA specimens from the benign prostatic glands also showed *TMPRSS2-ERG* fusion type A. These two cases were subjected to repeated pathological examination. However, based on morphology we could not identify malignancy in the areas sampled.

To determine whether the presence of *TMPRSS2-ERG* fusion type A in prostate cancer tissue was associated with disease progression, we compared PSA recurrence status between patients with or without fusion (Table 2). Among the 28 patients who had *TMPRSS2-ERG* fusion type A-positive tumors, 3 of 28 experienced PSA recurrence, whereas 4 of the 17 patients who had tumors without fusion transcript experienced PSA recurrence. There was no statistical difference between patients who had tumors with and without *TMPRSS2-ERG* fusion type A and PSA recurrence ($P = 0.3986$). This was also confirmed by Kaplan–Meier unadjusted survival analysis, which did not show significance ($P = 0.42$).

Figure 1 Sensitivity assay for *TMPRSS2-ERG* fusion and schematic representation of *TMPRSS2-ERG* fusion A and C transcripts. Serial dilutions of VCaP RNA were assayed for *TMPRSS2-ERG* fusion type A expression by quantitative RT-PCR. (a) Quantitative real-time RT-PCR plot. (b) Gel electrophoresis of the PCR products. (c) Exons are represented by numbered boxes. The arrow symbolizes the fusion.

We also examined whether the presence of *TMPRSS2-ERG* fusion transcripts, especially *TMPRSS2-ERG* fusion type A, correlated with other prognostic variables. All the parameters, including age at diagnosis, race, preoperative PSA, clinical stage, pathological stage, Gleason score, surgical margin, prostate size, and index tumor volume, did not correlate with the presence of *TMPRSS2-ERG* fusion type A (Table 2).

In a univariate Cox model, there was no relationship of the *TMPRSS2-ERG* fusion type A with various clinicopathologic variables; only Gleason score ($P=0.0405$) and index tumor

volume ($P=0.0132$) were significant predictors for PSA recurrence (Table 3).

In addition, we examined whether *TMPRSS2-ERG* fusion type A was frequently present in the index tumor by using Fisher exact test. For this analysis, a total of 27 prostatectomies were available, which contained at least both T1 (index or largest) and T2 (second largest) tumor in a given whole-mount section. *TMPRSS2-ERG* fusion type A was prevalent in 19 of 27 T1 compared to 8 of 27 T2. However, due to the small sample size, it was not statistically significant ($P=0.36$).

Table 1 Comparisons of *TMPRSS2-ERG* fusion pattern in separate tumors and non-malignant area from the same prostate

Case No.	N		PIN		T1		T2		T3		T4	
	A	C	A	C	A	C	A	C	A	C	A	C
1												
2												
3												
4												
5												
6												
7												
8												
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Tumors T1, T2, T3, and T4 designate separate tumors in the same prostate. Red = positive for *TMPRSS2-ERG* fusion; green = negative for *TMPRSS2-ERG* fusion; gray = not evaluable; A = *TMPRSS2-ERG* fusion type A; C = *TMPRSS2-ERG* fusion type C; N = non-malignant.

Discussion

Most prostates contain multi-focal tumors.^{1,16–19} The heterogeneity of prostate cancer in multi-focal tumor context has also been well documented. The question arises whether these multi-focal tumors in the same prostate are of independent origin. To address this question, we analyzed the common genomic alterations of the *TMPRSS2-ERG*, recently described

by several groups in clinically localized prostate cancer.^{6,20–41}

In the study by Gao *et al*,¹³ at least one tumor focus of 67% of patients exhibited *TMPRSS2-ERG* fusion, of which is similar to our recent finding of 62% in laser capture microdissected OCT-embedded frozen specimens. We found that half of the specimens collected from the index tumor contained this molecular alteration.

Figure 2 Topographical data of *TMPRSS2-ERG* fusion from six patients. Tumors T1, T2, and T3 designate separate tumors in the same prostate. PIN=prostatic intraepithelial neoplasia; + = positive for *TMPRSS2-ERG* fusion; - = negative for *TMPRSS2-ERG* fusion; A = *TMPRSS2-ERG* fusion type A; C = *TMPRSS2-ERG* fusion type C.

This study highlights the frequent presence of the *TMPRSS2-ERG* fusion transcripts in index tumors and suggests a critical role of *ERG* alterations in the onset and/or progression of a large subset of prostate cancer. *TMPRSS2-ERG* heterogeneity in separate tumor foci of the same patient further reinforces the concept of independent origin of multiple tumors, which had been addressed.^{1,42,43}

There was no statistical difference between patients who had tumors with and without *TMPRSS2-ERG* fusion type A and PSA recurrence ($P=0.3986$). The current data based on 45 patients show a similar trend as in our previous study based on frozen specimens.¹³ However, the lack of prognostic significance of *TMPRSS2-ERG* status may have been masked by the limited sample number.

Of 45 non-malignant samples, 2 showed *TMPRSS2-ERG* fusion. When these two cases were subjected to a repeated pathological examination, we could not identify PIN or carcinoma but some morphologically benign glands, some of which showed α -methylacyl-CoA racemase expression in luminal cells by immunohistochemistry. Previously, Leav *et al*⁴⁴ found that by using α -methylacyl-CoA racemase it is possible to define areas of transition from benign glands to malignant glands. Virtually, all of the *TMPRSS2-ERG* fusion reports show the gene fusion or the fusion transcripts in malignant glands.^{6,21–40} Clark *et al*⁴¹ detected several patterns of gene *TMPRSS2-ERG* fusion transcripts, including type A in 2 of 31 (6%) 'benign prostatic hyperplasia' and in 8 of 17 (43%) 'non-malignant specimens.' It is conceivable that

Table 2 Clinicopathologic correlation between *TMPRSS2-ERG* fusion-positive and -negative cases

Parameter	<i>TMPRSS2-ERG</i> fusion negative	<i>TMPRSS2-ERG</i> fusion positive	P-value
Age at surgery (years)	59.1 ± 7.8	57.4 ± 8.8	0.5068
Prostate size (cm ³)	52.2 ± 38.7	43.0 ± 21.6	0.6184 ^a
Index tumor volume (cm ³)	6.0 ± 9.4	4.5 ± 3.9	0.7963 ^a
<i>Race</i>			1
African American	3 (42.9%)	4 (57.1%)	
Caucasian	14 (36.8%)	24 (63.2%)	
<i>PSA (ng/ml)</i>			0.7401
<4.0	6 (40.0%)	9 (60.0%)	
4.0–10	7 (36.8%)	12 (63.2%)	
>10	3 (60.0%)	2 (40.0%)	
<i>Clinical stage</i>			0.731
T1	10 (40.0%)	15 (60.0%)	
T2–3	7 (35.0%)	13 (65.0%)	
<i>Pathological stage</i>			0.283
pT2	8 (47.1%)	9 (52.9%)	
pT3	7 (30.4%)	16 (69.6%)	
<i>Gleason score</i>			1
2–6	8 (38.1%)	13 (61.9%)	
7	7 (41.2%)	10 (58.8%)	
8–10	2 (33.3%)	4 (66.7%)	
<i>Surgical margin status</i>			0.277
Negative	13 (43.3%)	17 (56.7%)	
Positive	4 (26.7%)	11 (73.3%)	
<i>PSA recurrence status</i>			0.3986
No	13 (34.2%)	25 (65.8%)	
Yes	4 (57.1%)	3 (42.9%)	

^aP-value comes from *t*-test based on log transformation.

Table 3 Univariate Cox model for biochemical-free survival after surgery

Variables	Hazard ratio	95% confidence interval	P-value
Age at surgery (years)	1.134	0.352–3.650	0.8334
Race (Caucasian vs African American) ^a	—	—	—
Clinical stage (T2–3 vs T1)	2.12	0.388–11.578	0.3857
<i>Preoperative PSA (ng/ml)</i>			0.4554
4–10 vs <4	4.048	0.452–32.274	0.2114
>10 vs <4	3.442	0.215–55.117	0.3823
Pathologic stage (T3 vs T2)	3.59	0.401–32.156	0.2533
<i>Gleason score</i>			0.0405
7 vs 6	3.832	0.347–42.297	0.2729
8–10 vs 6	16.075	1.651–156.558	0.0168
Surgical margin (positive vs negative)	5.239	0.957–28.671	0.0562
Index tumor volume (cm ³)	1.088	1.018–1.164	0.0132
<i>TMPRSS2-ERG</i> fusion (positive vs negative)	0.508	0.102–2.518	0.4071

^aThere is no PSA recurrence in African-American men.

TMPRSS2-ERG fusion type A-positive benign glands may have the potential to develop into PIN or carcinoma. However, this hypothesis has to be further evaluated in a large cohort study.

We also found that a subset of PIN lesions (2 of 14 foci) showed *TMPRSS2-ERG* fusion type A. These PIN lesions with detectable *TMPRSS2-ERG* fusion may undergo further genetic changes to develop

prostate cancer. Linkage of PIN (high-grade PIN) and cancer has been well documented.⁴⁵

Conclusions

Information regarding the origin of multiple tumors in prostate cancer is critical to our understanding of the pathobiology and natural history of this disease. In this regard, prevalent oncogenic alterations of *ERG* due to *TMPRSS2-ERG* fusion in early-stage prostate cancer provide a break through observation to investigate the role of this potentially causal prostate cancer-specific genomic alteration in the origins of multi-focal prostate cancer. Our data show that half of the malignant areas in prostates harbor *TMPRSS2-ERG* fusions and apparent heterogeneity of *TMPRSS2-ERG* supports the independent origins of multi-focal tumors in the same prostate. Based on limited data, we suggest that foci of benign glands or PIN with *TMPRSS2-ERG* fusion type A may give rise to carcinoma. Thus, detection of benign glands with *TMPRSS2-ERG* fusion in biopsy or TURP specimen might suggest that these patients should be closely monitored.

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Conflict of interest

The authors indicated no potential conflict of interest. The views expressed in this article are those of the authors and do not reflect the official policy of the Department of the Army, Department of Defense, or the US Government.

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