Neoplasia • Vol. 8, No. 10, October 2006, pp. 885–888 885

www.neoplasia.com

Noninvasive Detection of *TMPRSS2:ERG* Fusion Transcripts in the Urine of Men with Prostate Cancer[1]

Bharathi Laxman[*,2], *Scott A. Tomlins*[*,2], *Rohit Mehra*[*,†], *David S. Morris*[‡], *Lei Wang*[*], *Beth E. Helgeson*[*], *Rajal B. Shah*[*,†,‡,§], *Mark A. Rubin*[¶,#], *John T. Wei*[†,‡,§] *and Arul M. Chinnaiyan*[*,†,‡,§]

*Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA; †Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI 48109, USA; ‡Department of Urology, University of Michigan Medical School, Ann Arbor, MI 48109, USA; §Michigan Urology Center, University of Michigan Medical School, Ann Arbor, MI 48109, USA; ¶Department of Pathology, Harvard Medical School, Boston, MA, USA; #Brigham and Women's Hospital, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA

Abstract

We recently reported the identification of recurrent gene fusions in the majority of prostate cancers involving the 5′ untranslated region of the androgen-regulated gene *TMPRSS2* and the *ETS* family members *ERG*, *ETV1*, and *ETV4*. Here we report the noninvasive detection of these gene fusions in the urine of patients with clinically localized prostate cancer. By quantitative polymerase chain reaction, we assessed the expression of *ERG* and *TMPRSS2:ERG* transcripts in urine samples obtained after prostatic massage from 19 patients (11 prebiopsy and 8 pre–radical prostatectomy) with prostate cancer. We observed a strong concordance between *ERG* overexpression and *TMPRSS2:ERG* expression, with 8 of 19 (42%) patients having detectable *TMPRSS2:ERG* transcripts in their urine. Importantly, by fluorescence *in situ* hybridization, we confirmed the presence or the absence of *TMPRSS2:ERG* gene fusions in matched prostate cancer tissue samples from three of three patients with fusion transcripts in their urine and from two of two patients without fusion transcripts in their urine. These results demonstrate that *TMPRSS2:ERG* gene fusions can be detected in the urine of patients with prostate cancer and support larger studies on prospective cohorts for noninvasive detection of prostate cancer.

Neoplasia (2006) **8,** 885–888

Keywords: Gene fusions, prostate cancer, noninvasive detection, urine, quantitative PCR.

Introduction

Chromosomal rearrangements play causal roles in numerous human malignancies and have been exploited diagnostically and therapeutically [1,2]. Using a novel bioinformatics strategy to nominate candidate oncogenes, we identified recurrent gene fusions involving the 5′ untranslated region of the androgen-regulated gene *TMPRSS2* to members of the *ETS* gene family (*ERG*, *ETV1*, or *ETV4*) in the majority of prostate cancers [3,4]. Subsequently, multiple studies have confirmed the presence of *TMPRSS2:ETS* gene fusions, particularly *TMRPSS2:ERG*, in 40% to 80% of prostate cancers [5–8]. In addition to likely playing a central role in the pathogenesis of prostate cancers, these studies highlight the potential of *TMPRSS2:ETS* gene fusions to serve as a specific biomarker of prostate cancer.

In an effort to develop a noninvasive method to detect *TMPRSS2:ERG* gene rearrangements, we explored the possibility of identifying this fusion in urine samples obtained from patients with prostate cancer using quantitative polymerase chain reaction (qPCR). Here we show that RNA isolated from sedimented urine and subjected to qPCR revealed the presence of *TMPRSS2:ERG* fusions in 8 of 19 (42%) patients with prostate cancer. We validated the specificity of this assay by confirming the presence or the absence of *TMPRSS2:ERG* gene rearrangements in matched tissue samples from a subset of our cohort. The results demonstrate the feasibility of the noninvasive detection of *TMRPSS2:ETS* gene fusions from the urine of patients with prostate cancer.

Materials and Methods

Urine Collection, RNA Isolation, and Amplification

This study was approved by the Institutional Review Board (IRB) of the University of Michigan Medical School (Ann Arbor,

Address all correspondence to: Arul M. Chinnaiyan, MD, PhD, Department of Pathology, University of Michigan Medical School, 1400 East Medical Center Drive 5316 CCGC, Ann Arbor, MI 48109-0602. E-mail: arul@umich.edu

[1]This work was supported, in part, by the National Institutes of Health Early Detection Research Network (UO1 CA111275-01 to A.M.C. and J.T.W.), the Prostate SPORE (P50CA69568 to K.JA.M.C. and R.B.S.), the Prostate Cancer Foundation (to A.M.C.), and the Department of Defense (PC040517 to R.M.). S.A.T. is supported by a Rackham Predoctoral Fellowship and is a fellow of the Medical Scientist Training Program. A.M.C. is supported by a Clinical Translational Research Award from the Burroughs Welcome Foundation.

[2]Co–first authors.

Received 3 October 2006; Revised 3 October 2006; Accepted 3 October 2006.

DOI 10.1593/neo.06625

MI). With informed consent of the patients, urine samples were obtained following a digital rectal exam before either needle biopsy or radical prostatectomy. Urine was voided into urine collection cups containing DNA/RNA preservative (Sierra Diagnostics LLC, Sonora, CA). For RNA isolation, a minimum of 30 ml of urine was centrifuged at 4000 rpm for 15 minutes at 4°C. RNAlater (Ambion, Inc., Austin, TX) was added to urine sediments and stored at −20°C until RNA isolation. Total RNA was isolated using an RNeasy Micro kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. RNA integrity was verified using an Agilent 2100 Bioanalyzer. Total RNA was amplified using an Omni-Plex Whole Transcriptome Amplification (WTA) kit (Rubicon Genomics, Ann Arbor, MI) according to the manufacturer's instructions, essentially as previously described [9]. Twenty-five nanograms of total RNA was used for WTA library synthesis, and cDNA library was subjected to one round of WTA PCR amplification. Amplified cDNA was purified using a QIAquick PCR Purification kit (Qiagen, Inc.). For cell line proof-of-concept experiments, the indicated number of VCaP or LNCaP cells was spiked into 1 ml of urine, and samples were processed like voided urine.

qPCR

qPCR was used to detect *ERG*, *ETV1*, and *TMPRSS2:ERG* transcripts from WTA-amplified cDNA, essentially as described [4]. For each qPCR, 10 ng of WTA-amplified cDNA was used as template. 2× Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 25 ng of both forward and reverse primers were used for *ERG*, *ETV1*, prostate-specific antigen (*PSA*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) qPCR. 2× *Taqman* Universal PCR Master Mix, a final concentration of 900 nM forward and reverse primers, and 250 nM probe were used for *Taqman TMPRSS2:ERGa*. For the *Taqman* assay, samples with C_t (threshold cycle) values greater than 38 cycles were considered to show no amplification. Threshold levels were set at the exponential phase of qPCR using Sequence Detection Software version 1.2.2 (Applied Biosystems). The amount of each target gene relative to the housekeeping gene *GAPDH* for each sample was determined using the comparative threshold cycle (C_t) method (Applied Biosystems user bulletin 2; http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). Samples with inadequate amplification of *PSA* (C_t > 22), indicating poor recovery of prostate cells in the urine, were excluded from further analysis. *ERG* (exons 5 and 6) and *ETV1* (exons 6 and 7) [4], *GAPDH* [10], and *PSA* [11] primers were as described. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). *Taqman* primers and probe (MGB-labeled, synthesized by Applied Biosystems) specific for *TMPRSS2:ERGa* are as follows:

TM-ERGa2_MGB-f: CGCGGCAGGAAGCCTTA
TM-ERGa2_MGB-r: TCCGTAGGCACACTCAAACAAC
TM-ERGa2_MGB-probe: 5′-MGB-CAGTTGTGAGT-GAGGACC-NFQ-3′.

Fluorescence In Situ *Hybridization (FISH)*

We acquired matched biopsy tissues from the University of Michigan Prostate Cancer Specialized Program of Research Excellence (SPORE) Tissue Core and prostatectomy tissue sections from the radical prostatectomy series at the University of Michigan, which is part of the SPORE. All samples were collected with informed consent of the patients and prior IRB approval. Formalin-fixed paraffin-embedded tissue sections were used for interphase FISH, as described [3,4]. For metaphase FISH on VCaP and LNCaP cells, metaphase spreads were prepared using standard methods. For analysis of *ERG* gene rearrangement, we used a split-signal probe strategy, with two probes spanning the *ERG* locus (5′, digoxin dUTP–labeled BAC clone RP11-95I21; 3′, biotin 14-dCTP–labeled BAC clone RP11-476D17). All BAC clones were obtained from the Children's Hospital of Oakland Research Institute.

Results and Discussion

We sought to develop a method to detect the presence of *TMPRSS2:ETS* fusion transcripts in prostate cancer cells shed into the urine after a digital rectal exam. As proof of concept, we employed urine spiked with prostate cancer cell lines expressing high levels of *ERG* and *TMPRSS2:ERG* (VCaP) or high levels of *ETV1* (LNCaP). As shown in Figure 1, we were able to detect *ERG* overexpression exclusively in VCaP at 1600 cells and *ETV1* overexpression exclusively in LNCaP at 16000 cells by qPCR. By correlating the number of spiked VCaP and LNCaP cells to *GAPDH* and *PSA* C_t values, we observed that urine obtained from patients after a digital rectal exam contained cell numbers insufficient to reliably detect *ERG* or *ETV1* overexpression (data not shown). Thus, we amplified total RNA collected from the urine of patients with prostate cancer using Omni-Plex WTA before qPCR analysis. We have previously validated WTA for RNA amplification before qPCR and/or DNA

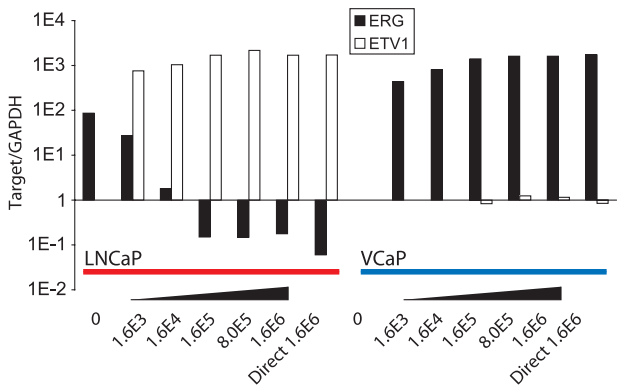

Figure 1. *Detection of ERG and ETV1 transcripts in urine spiked with prostate cancer cell lines. The indicated number of LNCaP (red bar: high ETV1 expression) or VCaP (blue bar: high ERG and TMPRSS2:ERG expression) prostate cancer cells was spiked into 1 ml of urine. Approximately 1.6 million cells of each cell line were used without being spiked (Direct). Total RNA was isolated and reverse-transcribed to cDNA before qPCR analysis. The relative amount of ERG and ETV1 for each sample was normalized to the amount of GAPDH.*

Table 1. Noninvasive Detection of *TMPRSS2:ERG* Gene Fusions in the Urine of Men with Prostate Cancer.

Sample ID	Type	Gleason Major	Gleason Minor	Gleason Score	*PSA* (ng/ml)	Age	*ERG*	Fusion (C_t)	FISH
LNCaP Cell line		NA	NA	NA	NA	NA	0.01	NQ	–
5778	Bx	3	3	6	11.7	52	NQ	NQ	–
5797	RP	3	4	7	5.3	52	NQ	NQ	–
5892	RP	4	3	7	8.9	57	NQ	NQ	
5909	Bx	3	3	6	7.8	56	0.04	NQ	
5918	Bx	3	3	6	3	56	0.06	NQ	
5915	Bx	3	4	7	6.9	71	0.20	NQ	
5798	RP	3	3	6	2.7	47	0.20	NQ	
5859	RP	3	3	6	8.7	63	0.27	NQ	
5893	RP	3	3	6	0.22	59	0.38	33.71	
5880	RP	3	3	6	2.96	67	0.40	NQ	
5796	Bx	4	5	9	19.3	82	0.98	NQ	
5780	Bx	3	3	6	5.9	79	1.00	NQ	
5794	Bx	3	3	6	3.8	56	1.09	38.96	
5864	RP	3	4	7	5.5	49	18.06	32.65	
5776	Bx	3	3	6	2.8	54	22.01	30.66	+
5775	Bx	3	3	6	5.99	62	30.91	32.87	
5815	RP	3	4	7	5.4	59	206.50	31.78	+
5790	Bx	3	4	7	5.5	51	328.56	31.48	+
5912	Bx	3	4	7	15.5	67	797.86	34.13	
VCaP Cell line		NA	NA	NA	NA	NA	226633.25	21.66	+

Each urine specimen was obtained from a unique patient assigned an ID, and urine samples spiked with 1.6 million VCaP or LNCaP cells were also assessed. The source of the sample, prebiopsy (Bx) or pre–radical prostatectomy (RP), is indicated. For all patients, major Gleason, minor Gleason, Gleason sum score, prebiopsy or preprostatectomy *PSA* (ng/ml), and age are reported. qPCR was used to measure the amount of *ERG* relative to *PSA* for each specimen. Samples were also assessed for the expression of *TMPRSS2:ERGa* using a specific *Taqman* assay, with positive samples indicated by the threshold cycle (C_t) of amplification. Matched prostate cancer tissue samples for five samples were assessed by FISH for *TMPRSS2:ERG* fusion using a split-probe assay for *ERG* rearrangement. Samples negative or positive for *TMPRSS2:ERG* rearrangements are indicated by (–) or (+), respectively. *NQ*, no quantifiable amplification of ERG; *ND*, no detectable amplification of *ERG* or *TMPRSS2:ERG* for the respective assays.

microarray analysis [9]. Using this strategy, we assessed two cohorts containing a total of 19 men with prostate cancer. After a digital rectal exam, urine was collected from 11 men before the performance of needle biopsy, which revealed the presence of prostate cancer. We also assessed a cohort of eight patients with prostate cancer from whom urine was collected after a digital rectal exam but before radical prostatectomy. Cohort characteristics are presented in Table 1.

For each patient, we determined the expression of *ERG* relative to *PSA*, in addition to determining whether the sample expressed *TMPRSS2:ERG* fusion transcripts. To confirm the specificity of our *TMPRSS2:ERG Taqman* primer/probe assay, we assayed urine samples spiked with 1.6 million LNCaP or VCaP cells. We detected *TMPRSS2:ERG* fusion transcripts exclusively in VCaP cells, which we have previously shown to markedly overexpress *ERG* and to harbor *TMPRSS2:ERG* rearrangement [4]. By this same assay, 8 of 19 (42%) urine samples expressed *TMPRSS2:ERG*, including the seven samples with the highest expression of *ERG* (Table 1). These results are consistent with previous studies demonstrating an overall frequency of 40% to 80% for *TMPRSS2:ERG* fusions in prostate cancer tissue samples and demonstrating that ~95% of samples with *ERG* overexpression harbor *TMPRSS2:ERG* gene fusions [4–8]. We did not detect *ETV1* overexpression in any sample.

As a confirmation of the specificity of our qPCR assay, we used FISH on matched tissue samples to determine the presence or the absence of the *TMPRSS2:ERG* gene rearrangement in the patient's prostate cancer. We used a split-probe FISH assay, with probes located 5′ and 3′ to the *ERG*, where a *TMPRSS2:ERG* gene rearrangement is indicated by splitting of one pair of probes or by loss of the 5′ *ERG* probe, which is consistent with an intrachromosomal deletion between *TMPRSS2* and *ERG* on chromosome 21q [4,5,8]. We expected that prostate cancer tissues from patients with high levels of *ERG* and *TMPRSS2:ERG* transcripts in their urine should be positive by FISH, whereas prostate cancer from patients with low levels of *ERG* and no detectable *TMPRSS2:ERG* transcripts in their urine should be negative by FISH. Thus, we assessed matched prostate tissue samples from three patients with detectable *TMPRSS2:ERG* in their urine and from two patients without detectable *TMPRSS2:ERG* in their urine. As expected, tissues from the three patients with high levels of *ERG* and detectable levels of *TMRPSS2:ERG* in their urine were positive for *ERG* rearrangement by FISH, whereas the two samples without *TMPRSS2:ERG* in their urine were negative for *ERG* rearrangement by FISH (Table 1). Hematoxylin and eosin–stained tissue sections and corresponding negative FISH assay from sample 5778, and a positive FISH assay from sample 5790 with deletion of the 5′ *ERG* probe are shown in Figure 2, *A–D*.

In summary, we have described the noninvasive detection of *TMPRSS2:ERG* fusion transcripts in the urine of patients with prostate cancer. We and others have recently described the presence of *TMPRSS2:ETS* gene fusions in the majority of prostate cancers and the utility of these gene fusions as a specific tissue biomarker of prostate cancer [3–8]. One limitation of the *TMPRSS2:ERG Taqman* assay we used for this study is that it only detects the *TMPRSS2:ERGa* isoform,

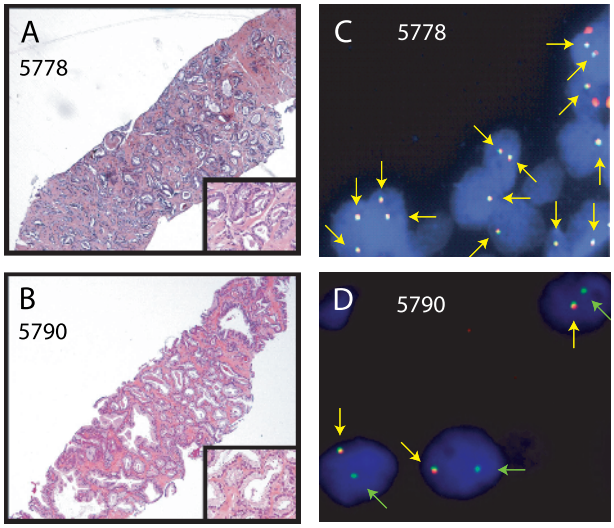

Figure 2. *Confirmation of the presence or the absence of TMPRSS2:ERG detection in the urine using FISH on matched tissue sections. Matched prostate cancer tissue samples for five samples were assessed by FISH for TMPRSS2:ERG fusion using a split-probe assay for ERG rearrangement (Table 1). Hematoxylin and eosin staining (A and B) and representative FISH images (C and D) for samples 5778 and 5790 are shown. A negative FISH assay (C) for sample 5778 is indicated by two pairs of colocalized red and green signals (yellow arrows) per cell, whereas a positive FISH assay is indicated by one pair of split red and green signals (not shown) or exclusive loss of the 5′ ERG probe (red signal) resulting in one pair of colocalized signals (yellow arrows) and one green signal (green arrows) per cell (D), as shown for sample 5790.*

which is expressed in approximately 85% to 95% of fusion-positive prostate cancers [4,7]. Thus, additional assays will be needed to detect alternative isoforms expressed in the remaining 10% to 20% of positive cases. Isoform-specific assays may be particularly relevant, as particular isoforms have been associated with aggressive disease [7]. The presence of prostate cancer cells in the sedimented urine of prostate cancer suggests that other approaches to detect *TMPRSS2:ETS* gene rearrangements, such as urine-based FISH similar to the UroVysion system for detecting bladder cancer [12], may also be feasible. A FISH-based assay would also be able to identify *TMPRSS2:ERG*$^{+}$ cases with intrachromosomal deletion between *TMPRSS2* and *ERG*, which has also been associated with aggressive disease in some cohorts [5,8]. In conclusion, the results reported herein support large-scale studies in prospective cohorts to determine the specificity and the sensitivity of urine-based assays for the detection of prostate cancer.

Acknowledgements

The authors thank Bo Han, Anjana Menon, and Alex Bond for technical assistance.

References

[1] Rabbitts TH (1994). Chromosomal translocations in human cancer. *Nature* **372,** 143–149.
[2] Rowley JD (2001). Chromosome translocations: dangerous liaisons revisited. *Nat Rev Cancer* **1,** 245–250.
[3] Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, Cao X, Wei JT, Rubin MA, Shah RB, et al. (2006). *TMPRSS2:ETV4* gene fusions define a third molecular subtype of prostate cancer. *Cancer Res* **66,** 3396–3400.
[4] Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, et al. (2005). Recurrent fusion of *TMPRSS2* and *ETS* transcription factor genes in prostate cancer. *Science* **310,** 644–648.
[5] Perner S, Demichelis F, Beroukhim R, Schmidt FH, Mosquera JM, Setlur S, Tchinda J, Tomlins SA, Hofer MD, Pienta KG, et al. (2006). *TMPRSS2:ERG* fusion–associated deletions provide insight into the heterogeneity of prostate cancer. *Cancer Res* **66,** 8337–8341.
[6] Soller MJ, Isaksson M, Elfving P, Soller W, Lundgren R, and Panagopoulos I (2006). Confirmation of the high frequency of the *TMPRSS2/ERG* fusion gene in prostate cancer. *Genes Chromosomes Cancer* **45,** 717–719.
[7] Wang J, Cai Y, Ren C, and Ittmann M (2006). Expression of variant *TMPRSS2/ERG* fusion messenger RNAs is associated with aggressive prostate cancer. *Cancer Res* **66,** 8347–8351.
[8] Yoshimoto M, Joshua AM, Chilton-Macneill S, Bayani J, Selvarajah S, Evans AJ, Zielenska M, and Squire JA (2006). Three-color FISH analysis of *TMPRSS2/ERG* fusions in prostate cancer indicates that genomic microdeletion of chromosome 21 is associated with rearrangement. *Neoplasia* **8,** 465–469.
[9] Tomlins SA, Mehra R, Rhodes DR, Shah RB, Rubin MA, Bruening E, Makarov V, and Chinnaiyan AM (2006). Whole transcriptome amplification for gene expression profiling and development of molecular archives. *Neoplasia* **8,** 153–162.
[10] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, and Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3,** RESEARCH0034.
[11] Specht K, Richter T, Muller U, Walch A, Werner M, and Hofler H (2001). Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am J Pathol* **158,** 419–429.
[12] Halling KC (2003). Vysis UroVysion for the detection of urothelial carcinoma. *Expert Rev Mol Diagn* **3,** 507–519.