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Detection of *TMPRSS2-ERG* Fusion Gene Expression in Prostate Cancer Specimens by a Novel Assay using Branched DNA

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Abstract

Objectives: Genetic rearrangement of *TMPRSS2* regulatory sequences and coding sequences of the *ERG* gene has been detected in nearly half of prostate cancers. Quantitative assays to detect such *TMPRSS2-ERG* gene fusion have been limited to real time PCR techniques that rely on reverse transcriptase-based amplification. We sought to develop a novel assay that uses branched DNA (bDNA) technology to measure *TMPRSS2-ERG* fusion.

Methods: Branched DNA probes were designed to detect *TMPRSS2-ERG* gene fusion in prostate cancer cell lines. Non-quantitative, nested reverse transcription (RT)-PCR and fluorescence *in situ* hybridization (FISH) were used to ascertain *TMPRSS2-ERG* gene fusion status in prostate tissues.

Results: The branched DNA assay detected *TMPRSS2-ERG* gene fusion from less than 200 picogram of prostate cancer RNA, whereas more than 600 picogram of RNA was required for fusion gene detection by one step real time RT-PCR. In evaluation of clinical prostatectomy specimens, the branched DNA assay showed concordant detectable fusion signal in all 9 clinical samples that had fusion detected by nested RT-PCR or FISH. Moreover, branched DNA detected gene fusion in 2 of 16 prostate cancer tissue specimens that was not detected by FISH nor nested RT-PCR.

Conclusions: Our findings demonstrate a branched DNA assay that is effective for detection of *TMPRSS2-ERG* gene fusion in prostate cancer clinical specimens, thus providing an alternative method to ascertain the *TMPRSS2-ERG* gene fusion in human prostate cancer tissue.

Keywords

branched DNA; prostate cancer; ERG; biomarker

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INTRODUCTION

Tomlins et al. have reported a recurrent fusion of the androgen-regulated gene *TMPRSS2* to the ETS transcription factors *ERG*, *ETV1*, and *ETV4* in prostate cancer.^{1,2} Subsequently, multiple studies have confirmed the presence of *TMPRSS2-ETS* gene fusions, especially *TMPRSS2-ERG* as the most common, which is present in approximately 50% of prostate cancers.³⁻⁶ *ERG* has been previously identified as an overexpressed gene in prostate cancer by real time PCR using primers from 3' portion of the *ERG* gene, distal to the region involved by the *TMPRSS2* fusion with demonstrated increase of *ERG* mRNA expression in 62% of prostate cancers.⁷ Wang et al. further correlated the isoforms of *TMPRSS2-ERG* fusions and expression levels with clinical outcome in prostate cancers from patients undergoing radical prostatectomy.⁵ *TMPRSS2-ERG* fusion transcripts thus also have the potential to serve as a biomarker for a more aggressive clinical course of prostate cancer. Moreover, *TMPRSS2-ERG* fusion transcripts can be detected in urine obtained from patients with PCa after prostatic massage, suggesting possible avenues for clinical use of *TMPRSS2-ERG* fusion detection.⁸

Assays for detecting *TMPRSS2-ERG* fusion have been limited to those based on RT-PCR or FISH. RT-PCR requires the presence of a stable, full-length transcript that can be difficult to retain in routine clinical processing, whereas FISH requires subspecialty molecular pathology expertise that is not uniformly available. In contrast, branched DNA techniques for detecting expressed transcripts use in-solution hybridization followed by cooperative hybridization between a target RNA and a target-specific probe set in an assay platform that requires neither reverse transcription and target amplification (as required by RT-PCR) nor esoteric expertise (as required to interpret FISH in situ).^{9,10} Furthermore, bDNA is apparently less dependent on RNA quality than RT-PCR.¹¹ We therefore developed a novel branched DNA probe design to measure the *TMPRSS2-ERG* fusion mRNA expression in human prostate cancer specimens.

MATERIALS AND METHODS

Cell line

Vcap cells were purchased from American Type Culture collection (ATCC, Manassas, VA).

Sample selection

Radical prostatectomy tissue samples were obtained from the IRB approved Hershey Foundation Prostate Cancer Serum and Tumor Bank at Beth Israel Deaconess Medical Center (BIDMC). Morphologic diagnosis was performed by a pathologist on hematoxylin and eosin (H&E) stained slides made from both sides of the corresponding (OCT)-embedded prostatectomy tissue blocks using an optimal cutting temperature. OCT blocks containing more than 30% of prostate cancerous tissue (with Gleason Score of 6 or 7) were selected for RNA purification.

RNA purification

A biopsy punch (Miltex Inc., York, PA) was used to select the prostate cancer tissues from the OCT sample blocks. Benign or prostate cancer tissues were homogenized using a TissueLyser (Qiagen, Valencia, CA) at 28 Hz for 5 min. Total RNA was isolated using TRIzol reagent (Invitrogen Corporation, Calsbad, CA) according to the manufacturer's instructions. RNA integrity was verified using an Agilent 2100 Bioanalyzer. Samples with high RNA integrity number (RIN) > 7 were selected for analysis.

Nested RT-PCR and real-time PCR

Primers for *TMPRSS2-ERG* fusion detection by nested RT-PCR were *TMPRSS2-1F* 5'-CGCGAGCTAAGCAGGAGGCG-3', *ERG-541R* 5'-

TCATGTTTGGGGGTGGCATGTG-3', TMPRSS2-20F 5'-GGAGGCGGAGGCGGAGGG-3' and ERG-450R 5'-TTGGCCACACTGCATTCATCAGGA-3'. 1 µl of cDNA template was amplified in a final volume of 50 µl using Platinum Tag DNA Polymerase (Invitrogen Corporation, Calsbad, CA) according to the manufacture's instructions. 2 µl of first round PCR product was used as template for nested PCR (primers: TMPRSS2-20F and ERG-450R). SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for one-step real-time RT-PCR analysis on Applied Biosystems 7900HT Prism instrument. Primers for TMPRSS2-ERG fusion detection by real time PCR were TMPRSS2-1F 5'-CGCGAGCTAAGCAGGAGGCG-3', ERG-541R 5'-TCATGTTTGGGGGTGGCATGTG-3'. 50 µl of cDNA was synthesized from 10 ng total RNA and 1 µl cDNA is analyzed by real time PCR (two-step real-time PCR). Samples are performed in triplicate. Relative quantification analysis was used. Expression values of each gene were normalized to the expression of GAPDH of a given sample. The $2^{-\Delta\Delta C_t}$ method (available online from Applied Biosystem) was used to calculate relative expression of each gene.

Fluorescence in situ hybridization (FISH) analysis

FISH analysis of the specimens was performed as previously described by Perner and Mosquera.^{1,3,12} There were two differentially labeled probes designed to span the telomeric and centromeric neighboring regions of the *ERG* locus. A nucleus without *ERG* rearrangement gives a yellow signal (juxtaposed red and green signals). *TMPRSS2-ERG* fusion through insertion results in a single red and green signal for the rearranged *ERG* allele and a yellow non-rearranged allele in each nucleus. *TMPRSS2-ERG* fusion through deletion results in a loss of the green telomeric signal with a remaining red signal for the rearranged allele and a yellow signal for the non-rearranged allele.

Branched DNA (bDNA)

Experiments addressing optimal capture extender (CE), blocker (BL), and label extender (LE) concentrations indicate that these are 25 fmol, 50 fmol, and 100 fmol respectively across a broad range of bDNA assays. 10 ng of RNA were mixed with the working probe set and lysis mixture according to the QuantiGene 2.0 Reagent System manual (Panomics, Fremont, CA). 100 µl/well were then dispensed into the capture plate. Hybridization was performed overnight (16-20 hr) at 55°C and the plate was then processed following the protocol. the capture plate was read on Victor3 1420 multilabel counter (Perkin Elmer, Inc. Waltham, MA).

Statistical analysis

Detection sensitivities of real-time RT-PCR and bDNA technologies spiked in total RNA from Vcap cells were each determined by ANOVA modeling with pairwise comparisons of each dilution level versus the control. The *TMPRSS2-ERG* fusion gene expression measured by real-time RT-PCR and bDNA were compared between cancer and controls using Wilcoxon rank sum test. In addition, signal strength of each cancerous sample was compared to the 95% confidence limits of the mean of benign samples, and signals that exceeded control 95% confident limits were designated as indicating significant expression.

RESULTS

Branched DNA (bDNA) probe sets for the fusion gene were designed to capture the 5' portion of the *TMPRSS2* gene and exons 5-6 of the *ERG* gene (Figure 1). Sequence information regarding capture extender and label extender are shown in Figure 1. To compare the detection sensitivity of the *TMPRSS2-ERG* fusion by real-time RT-PCR and bDNA technologies, RNA from Vcap cells, known to express the *TMPRSS2-ERG* fusion, was combined with total RNA from benign human prostate to a total of 10 ng RNA. First, we tested the sensitivity of the bDNA technology by using an ANOVA model (Figure 2A). *TMPRSS2-ERG* fusion was

detected by significant luminescence ($p < 0.05$), at 0.156 ng or higher amounts of VCAP RNA (Figure 2A). The sensitivity of real time RT-PCR for detection of the *TMPRSS2-ERG* fusion is shown in Figure 2B and was limited to 0.625 ng or higher amounts of Vcap RNA ($p < 0.05$).

We next sought to determine if the increase in assay sensitivity, in terms of RNA amount required to detect fusion, would translate to improving the ability to detect *TMPRSS2-ERG* fusion in 20 clinical prostatectomy specimens. We first evaluated 4 benign and 16 cancerous prostatectomy samples by nested RT-PCR to determine their fusion status; a subset of the cancers' fusion status was ascertained by FISH detection of translocation or deletion (Table 1; insufficient sample tissue blocks precluded conclusive FISH assay in 5 cases). By nested RT-PCR, 9 of the 16 cancerous samples were fusion-positive (all 4 benign samples were fusion negative; Table 1). In 2 cases (cases 04-33LPB1-T3 and 04-33LPB4-T3) evaluable by FISH, FISH did not detect fusion that was detected by nested RT-PCR.

After ascertainment of fusion status by the non-quantitative nested RT-PCR technique, we next turned to a comparison of the sensitivity of real time PCR and branched DNA assays in detecting *TMPRSS2-ERG* gene fusion. Real-time RT-PCR failed to detect *TMPRSS2-ERG* gene fusion in 1/9 cancer cases that had fusion detectable by nested RT-PCR (case 04-34RB1-T1; Table 1). In contrast, the branched DNA assay detected *TMPRSS2-ERG* gene fusion in 9/9 cases that had been detected by nested RT-PCR and also detected the fusion in 2/16 cases that had not been detected by nested RT-PCR at 95% CL (cases 04-26L2 and 05-04RA1; Table 1).

DISCUSSION

Identifying specific cancer target genes is of paramount importance for early detection of the disease and the diagnosis of disease progression. To date, two common chromosomal aberrations have been identified in prostate cancer; the androgen receptor (*AR*) gene at Xq12 and *TMPRSS2-ERG* at 21q.¹³ with *TMPRSS2-ERG* fusion gene being the most common genetic aberration present in about half of clinically localized prostate cancer tissues.^{3-6,14}

Reverse transcription-PCR (RT-PCR), nested RT-PCR, RT-PCR followed by Southern blot hybridization with a radio labeled probe and FISH have been used to analyze the prostate cancer specific fusion genes.^{1,4,5,15} However, the sensitivity to detect *TMPRSS2-ERG* expression level using standard methods has not rigorously been evaluated. Currently, real time RT-PCR is the principal method for quantitative measurement of *TMPRSS2-ERG* fusion based on expression levels. Here we employed a novel probe set design based on the bDNA technology to detect the expression of the *TMPRSS2-ERG* fusion gene. The branched DNA assay described herein detected fusion transcripts at levels as low as 0.16 ng RNA. This sensitivity translated to clinical samples in the branched DNA assay successfully detecting fusion in all 9 positive prostate cancer samples found to have fusion by other non-quantitative methods (nested RT-PCR or FISH). Moreover, the branched DNA technique was able to detect *TMPRSS2-ERG* fusion in cases for which the standard FISH, RT-PCR, and real time PCR assays were not sufficiently sensitive to detect fusion. Possible reasons why the bDNA assay may detect *TMPRSS2-ERG* gene rearrangements not detected by nested RT-PCR include: 1) a gene rearrangement may occur outside of the nested RT-PCR primer sets, whereas the bDNA extender sequences flank beyond the internal nested primer sets by 185 base pairs; 2) the number of fusion-positive cells in a tissue specimen may be below the detection limits of nested RT-PCR but still be detected by bDNA due to the favorable inter-assay variance characteristics of bDNA-based techniques that do not rely on target gene amplification; 3) there were no real fusions in the two samples detected only by the branched DNA technique (i.e., these were false positive results). Only with additional detailed molecular studies will the true sensitivity and false positive rate of the branched DNA technique be determined. Concurrent studies suggest that branched DNA techniques in general may be applicable to detect gene rearrangement

directly from formalin fixed, paraffin embedded tissues without any need for distinct RNA isolation (extraction).¹¹

The differential expression of the fusion genes may provide useful information in clinical practice. Although the exact mechanism of *TMPRSS2-ERG* fusion is yet to be unraveled, this gene fusion is considered to be an early event in prostate cancer development.¹⁶ Under physiological androgenic stimulation, the fusion gene may lead to overexpression of *ERG* regulated genes involved in cell growth and differentiation.¹⁷ There is a significant link between *TMPRSS2-ERG* and a distinct phenotype in prostate cancer such as blue-ring mucin, cribriform growth pattern, macronucleoli, intraductal tumor spread, and signet-ring cell features.¹² The fusion gene also occurs in a proportion (21%) of high-grade prostatic intraepithelial neoplasia (HGPIN) lesions, possibly preceding chromosome copy number changes in prostate carcinomas, and has been associated with invasion.^{16,18} In a pool of 26 patients who underwent surgery for clinically localized prostate cancer, patients with the fusion gene had a significant higher rate of recurrence (5-year recurrence rate of 79.5%) compared to patients without the fusion gene (five-year recurrence rate of 37.5%).¹⁹ The *TMPRSS2-ERG* rearrangement has been implicated with prostate cancer clinical aggressiveness, but such an association has not been definitively confirmed.^{5,20,21} The *TMPRSS2-ERG* fusion was also reported to be associated with lethal prostate cancer in a watchful waiting cohort (cumulative incidence ratio = 2.7, $p < 0.01$, 95% confidence interval = 1.3 – 5.8).²⁰ Furthermore, a study showed that *TMPRSS2-ERG* fusion was observed in androgen receptor (AR)-negative xenograft and in clinical prostate cancer specimens, while the fusion gene was not expressed. However, two other wild-type ETS family genes, *ETV4* or *FLII*, were over-expressed in these AR-negative tumor samples.²²

In addition to the probable role the *TMPRSS2-ERG* fusion plays in the pathogenesis of prostate cancer, it also has the potential to serve as a biomarker of prostate cancer with more aggressive behavior as compared to the fusion negative prostate cancers. In this scope, *TMPRSS2-ERG* fusion transcripts have been detected in urine obtained after prostatic massage.⁹ Through a combination of *TMPRSS2-ERG* and prostate cancer antigen 3 (*PCA3*) assay, detection of transcripts in urinary sediments could achieve a sensitivity of 73% in a prostate cancer positive biopsy group. Given the specificity of *TMPRSS2-ERG* for prostate cancer, the sensitivity of prostate cancer diagnosis could be significantly improved. A positive detection for *TMPRSS2-ERG* fusion could prevent repeat biopsies of those patients with elevated serum PSA levels and a history of negative biopsies. Two out of 30 men with a prostate cancer-negative biopsy (7%) had detectable *TMPRSS2-ERG* fusion transcripts in their urinary sediments.¹⁵ Whether the branched DNA technique for detecting *TMPRSS2-ERG* fusion can be applied to readily accessible clinical specimens such as urine awaits further study.

CONCLUSION

With *TMPRSS2-ERG* fusion gene being the most common genetic aberration in prostate cancer, *TMPRSS2-ERG* fusion defines a subtype of prostate cancer patients. The branched DNA method described herein is an effective method for detection of *TMPRSS2-ERG* fusion gene expression in prostate cancer tissue samples that allows fusion to be identified. This novel technique may help optimize detection of *TMPRSS2-ERG* gene fusion and thereby provides a potentially useful tool for prostate cancer molecular staging and detection.

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Figure 1. Schematic illustration of the bDNA probe set for *TMPRSS2-ERG* fusion
 Probe sets for the fusion gene are designed to capture the 5' portion of the *TMPRSS2* gene and exons 5-6 of *ERG* gene and to quantify the expression of the eight known *TMPRSS2-ERG* subtypes.⁵ The capture extender (CE) probes are within exons 1-3 (1-365bp) of the *TMPRSS2* gene (NM_005656) and the label extender probes are within exons 5 and 6 (453-652bp) of the *ERG* gene (NM_004449). There is one capture extender at the end of the *ERG* gene (653-673bp). Three capture extenders are sufficient to capture >95% of RNA out of solution onto the capture well. The capture extender (CE) pool contains oligonucleotides that are complementary to the target RNA and to capture probes (CPs) covalently linked to a solid support either a plate or microsphere. The CE nucleotide sequences before TTTTT are

complementary to TMPRSS2 and ERG genes whereas the sequences that follow TTTTT are complementary to capture probes (and therefore mediate 'capture' or binding of the target gene to the assay plate). The label extender (LE) pool has oligonucleotides that contain sequences complementary to the target RNA and to the bDNA amplifier molecule. The sequences of LEAs and LEBs that precede TTTTT are complementary to ERG exon 5 and 6, whereas those that follow TTTTT are complementary to the bDNA amplifier molecule (that amplifies and links the signal to an alkaline phosphatase conjugate that, after addition of dioxetane chemiluminescence substrate, generates a luminescent signal that is proportional to the amount of target RNA present in the sample). The blocker (BL) pool contains oligonucleotides that are complementary to the target RNA where neither CE nor LE hybridizes and protects the target RNA by maintaining an intact RNA–DNA hybrid.²² The blocker sequences are available from the authors upon request.

Figure 2. Sensitivity of *TMPRSS2-ERG* expression by branched DNA and Real Time RT-PCR in total RNA from Vcap cells spiked with total RNA from normal human prostate tissue (A) Fusion gene expression detected by branched DNA. Triplicate wells are assessed for each sample. (B) Fusion gene expression measured by real time RT-PCR. A total of 10 ng RNA for each sample composed of Vcap RNA spiked with human normal prostate tissue RNA. Duplicate wells for each sample. Bar with a “*” indicates $P < 0.05$, and “**” $P < 0.01$ for comparison to control group which contains 0 ng Vcap RNA (only the lowest Vcap level that was different from control with $P < 0.01$ was labeled with “**”).

TMPRSS2-ERG fusion status evaluated by nested RT-PCR, real-time PCR, and branched DNA assay in 20 human prostate normal and cancerous samples

Table 1

	Normal prostate										Prostate Cancer									
	01-29LM	01-32-LM	01-28LM	01-44L	105-05LM	405-23LM	104-30L	A103-25R	204-26L	205-04RA	104-39RB	105-21RB	104-34RB	1-T104-33RP	PB2-T205-19R	101-17RB	203-18R	204-33LP	B1-T304-33LP	B4-T305-17RB
RIN*	7.2	8.2	7.4	8.1	7.7	7	7.3	8.6	7.8	7.4	7.8	7.9	8.7	7.3	7.2	8	7.2	7.8	7.6	8.3
Nested RT-PCR	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Real-time PCR	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+
FISH	N/A	N/A	N/A	N/A	-	-	-	-	-	N/A	-	+	N/A	N/A	+	N/A	+	-	-	N/A
Branched DNA	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+

+ *TMPRSS2-ERG* fusion positive; - *TMPRSS2-ERG* fusion negative

Criteria for detection of *TMPRSS2-ERG* fusion by real-time PCR or branched DNA assay included: consistent detectable fusion signal in triplicate samples and signal intensity higher than non-cancer control tissue (see Methods-Statistical Analysis).

* RNA integrity number;