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# Three-Color FISH Analysis of *TMPRSS2/ERG* Fusions in Prostate Cancer Indicates That Genomic Microdeletion of Chromosome 21 Is Associated with Rearrangement[1]

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## Abstract

**The recent description of novel recurrent gene fusions in ~80% of prostate cancer (PCa) cases has generated increased interest in the search for new translocations in other epithelial cancers and emphasizes the importance of understanding the origins and biologic implications of these genomic rearrangements. Analysis of 15 PCa cases by reverse transcription–polymerase chain reaction was used to detect six *ERG*-related gene fusion transcripts with *TMPRSS2*. No *TMPRSS2/ETV1* chimeric fusion was detected in this series. Three-color fluorescence *in situ* hybridization confirms that *TMPRSS2/ERG* fusion may be accompanied by a small hemizygous sequence deletion on chromosome 21 between *ERG* and *TMPRSS2* genes. Analysis of genomic architecture in the region of genomic rearrangement suggests that tracts of microhomology could facilitate *TMPRSS2/ERG* fusion events.**

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**Keywords:** *TMPRSS2/ERG* rearrangements, microhomology, genome architecture, FISH, genomic microdeletions.

## Introduction

As the most common neoplasm and second leading cause of cancer mortality in North American men [1], remarkably little is known about crucial events in prostatic carcinogenesis. Two related discoveries over the last 12 months, however, have the potential to considerably contribute to knowledge in this area. Initially, Petrovics et al. [2] described an ETS-related gene (*ERG1*) in the prostate cancer (PCa) transcriptome, suggesting that it was the most commonly overexpressed proto-oncogene in malignant prostatic tissues. Independently, Tomlins et al. [3] described novel gene fusions involving either *ERG1* or a related gene *ETV1* or *ETV4* [4], which is thought to underlie the mechanism of overexpression. These studies used a novel computational analysis of microarray data called Cancer Outlier Profile Analysis (COPA) and was subsequently confirmed by reverse transcription–polymerase chain reaction (RT-PCR) and fluorescence *in situ* hybridization (FISH). A confirmatory study also detected *ERG* rearrangements but did not detect *ETV* alteration [5]. The gene fusions described to date involve the androgen-sensitive *TMPRSS2* gene and three aforementioned members of the *ETS* family of transcription factors (*ERG*, *ETV1*, and *ETV4*). We provide independent confirmation of translocation results in 6 of 15 (40%) PCa specimens and additionally describe two novel variant transcripts in the same multicentric tumor. In addition, break-apart three-color FISH was used to confirm that a deletion between *TMPRSS2* and *ERG* on chromosome 21 was associated with gene fusion events.

## Materials and Methods

Fifteen typical PCa tissue samples were obtained from radical prostatectomies. Part of the tissue was embedded in frozen section medium and stored at −80°C until a tumor-rich tissue had been selected for RNA extraction. FISH analysis was performed on adjacent sections. Tissue sections were also stained with hematoxylin and eosin and subjected to standard histopathological evaluation to determine pathological grade, tumor

Abbreviations: aCGH, array-based comparative genomic hybridization; BAC, bacterial artificial chromosome; PCa, prostate cancer; COPA, Cancer Outlier Profile Analysis; FISH, fluorescence *in situ* hybridization; HPIN, high-grade prostatic intraepithelial neoplasia; PCR, polymerase chain reaction; PNT, pointed; PSA, prostate-specific antigen; RT-PCR, reverse transcription–polymerase chain reaction

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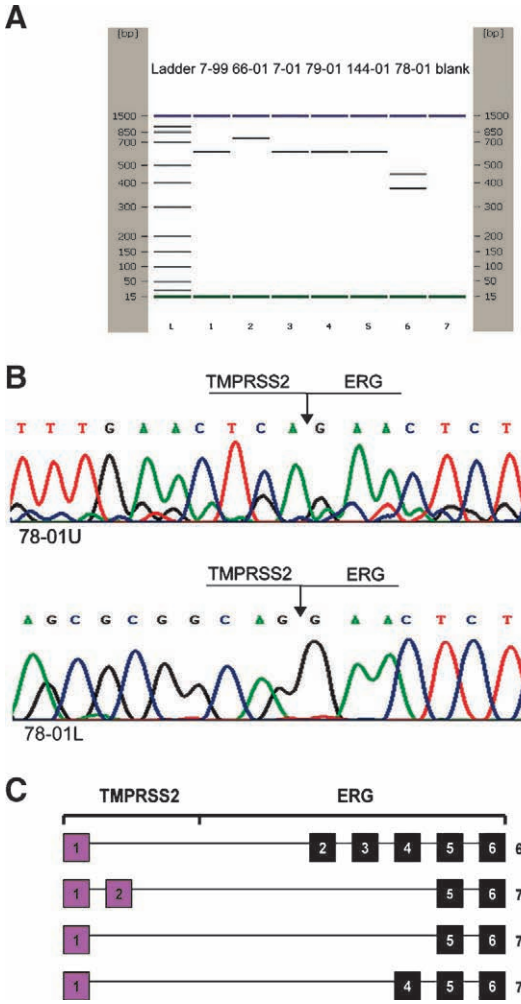


**Figure 1.** *Rearrangement of the TMPRSS2 and ERG genes in PCa. (A) RT-PCR products from six PCa cases were sized using the Agilent 2100 Bioanalyzer. The fragments were analyzed with a ladder marker to determine the size of each variant TMPRSS2/ERG transcript. Depending on the breakpoints within each, the fragments were 800, 600, ~430, and ~350 bp. (B) Sequence electropherograms of mutant TMPRSS2/ERG transcripts from case 78-01. Two unique variant transcripts were found to be present in this case: one containing exons 1 and 2 of the TMPRSS2 gene and exons 5 and 6 of the ERG gene, and the other containing exon 1 of the TMPRSS2 gene joined to exons 5 and 6 of the ERG gene. The arrows indicate gene breakpoints. (C) Schematic representation of the exon composition of the TMPRSS2/ERG gene fusion products from variant PCa cases.*

content, and the presence or absence of single/multifocal PCa. Gleason scores ranged from 6 to 9, and one tumor sample (78-01) was considered to have multicentric histology. To determine the prevalence of *ETS* rearrangement, RT-PCR amplification (GeneAmp RNA PCR Core Kit; Applied Biosystems, Foster City, CA) was carried out as described by Tomlins et al. [3]. Duplicated RT-PCR products from 15 PCa cases were sized by electrophoresis on a 1.5% agarose gel and by DNA 1000 LabChip Kit (Agilent 2100 Bioanalyzer; Agilent Technologies, Inc., Palo Alto, CA). These products were then gel-purified and sequenced directly using an ABI PRISM 377 (Applied Biosystems) sequencer (Figure 1, *B* and *C*).

To confirm the presence of *TMPRSS2/ERG* fusions, we used interphase FISH assays on corresponding frozen sections. A break-apart FISH strategy was employed in the analysis of *ERG* gene rearrangement using bacterial artificial chromosome (BAC) DNA probes published previously [3]. This approach consisted of two DNA probes positioned at opposite sides of the breakpoint region of the *ERG* gene (*ERG* 5′ and *ERG* 3′ loci) and differential labeling using the Ulysis Nucleic Acid Labeling kit (Molecular Probes, Eugene, OR). The OregonGreen-labeled RP11-95I21 BAC probe spans the *ERG* 5′ and extends inward into exon 10. The red-fluorescein–labeled RP11-476D17 BAC probe spans the *ERG* 3′ locus and extends inward past exon 4. There is a 35-kb gap between the 3′ and 5′ *ERG* probes. The *TMPRSS2* gene was identified using the Pacific Blue–labeled RP11-35C4 BAC probe, which starts 2.7 Mb from the 5′ end of the *ERG* gene (Figure 2). The FISH criteria used to evaluate *TMPRSS2/ERG* rearrangement were as follows: [1] visualization of separate green 5′ *ERG* and red 3′ *ERG* signals, and [2] enumeration of each green, red, and blue signal.

DAPI-stained tumor nuclei (dark blue) were identified in an adjacent H&E–stained frozen tissue. Normal signal patterns of the probes were confirmed by the colocalization of OregonGreen-labeled 5′ *ERG* (green signals), AlexaFluor 594–labeled 3′ *ERG* (red signals), and Pacific Blue–labeled *TMPRSS2* (pale blue signals) in normal peripheral lymphocyte metaphase cells and in normal interphase cells (Figure 3*A*). *ERG* rearrangement was confirmed by the split of one of the colocalized signals, in addition to a fused signal of the unaffected chromosome 21 (Figure 3*B*). A minimum of 300 signals per probe was counted to confirm the *TMPRSS2/ERG* rearrangement in PCa specimens previously analyzed by RT-PCR. A decreased ratio for the 5′ *ERG* probe mapping to the genomic interval between the

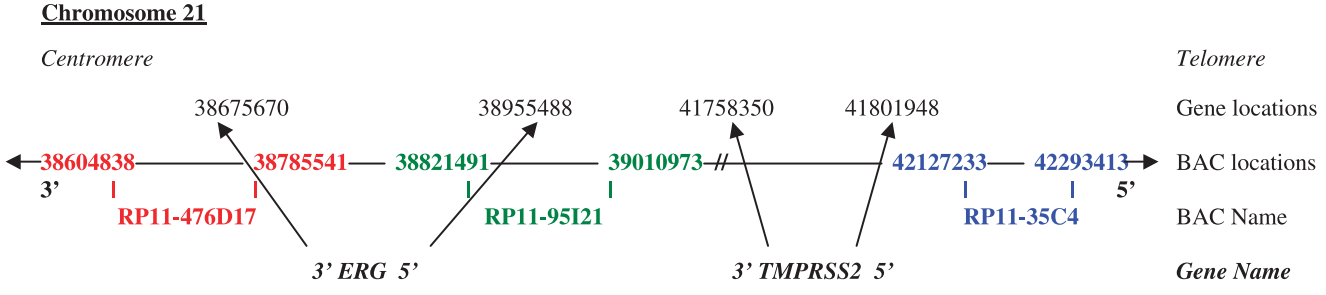


**Figure 2.** *Location and names of the BAC probes and gene locations used in the analysis. Gene locations are taken from the May 2004 assembly of the UCSC Genome Browser. Numbers indicate basepair location along the chromosome. Colors correspond to fluorochromes used in FISH experiments.*

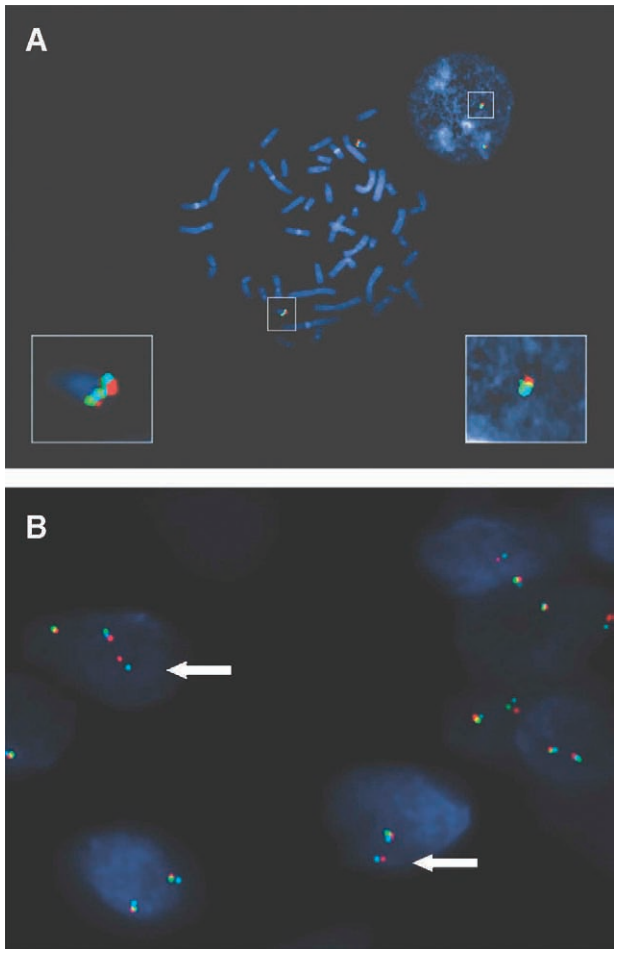


**Figure 3.** *FISH analysis showing rearrangement of TMPRSS2 and ERG genes in PCa. (A) FISH confirms the colocalization of OregonGreen-labeled 5′ ERG (green signals), AlexaFluor 594–labeled 3′ ERG (red signals), and Pacific Blue–labeled TMPRSS2 (light blue signals) in normal peripheral lymphocyte metaphase cells and in normal interphase cells. (B) In PCa cells, break-apart FISH results in a split of the colocalized 5′ green/3′ red signals, in addition to a fused signal (comprising green, red, and blue signals) of the unaffected chromosome 21. Using the TMPRSS2/ERG set of probes on PCa frozen sections, TMPRSS2 (blue signal) remains juxtaposed to ERG 3′ (red signal; see white arrows), whereas colocalized 5′ ERG signal (green) is lost, indicating the presence of TMPRSS2/ERG fusion and concomitant deletion of 5′ ERG region.*

*TMPRSS2* region and 3′ *ERG* (Figure 2) was indicative of hemizygous deletion. These experiments were optimized using FISH ratios present in normal adjacent tissues, and deletion cutoff values were defined as a ratio of green 5′ *ERG* signal to red 3′ *ERG* ($\leq$ 0.80) [6] when break-apart FISH analysis indicated that a fusion genomic rearrangement was present.

## Results and Discussion

Of the 15 tumors analyzed, 6 (40%) possessed an *ERG* rearrangement, confirming the FISH findings of a previous study (55%; 16 of 29) [3]. Although none of our samples had an *ETV1* rearrangement, this observation is not surprising because the original paper only detected the *ETV1* fusion transcript in a smaller proportion of samples (25%; 7 of 29) and because the confirmatory study by Soller et al. [5] did not detect *ETV1* alteration in 18 tested samples. Using the Agilent 2100 Bioanalyzer, fragment lengths were precisely determined. Five of six positive *ERG*/*TMPRSS2* fusions had lengths consistent with published findings; however, one tumor sample (PCa 78-01) contained two variant *TMPRSS2/ERG* transcripts (Figure 1*A*, *lane 6*) of 430 and 350 bp. Automated DNA sequencing of gel-purified transcripts from PCa 66-01 and PCa 79-01 (both typical *TMPRSS2/ERG* fusions) and from PCa 78-01 (upper and lower fragments) confirmed the fusion of *TMPRSS2* with the *ERG* gene. Sequence analysis of both gel-purified fragments from PCa 78-01 revealed two distinct in-frame rearrangements generating novel *TMPRSS2/ERG* fusion transcripts. The variant *TMPRSS2/ERG* transcript of 430 bp resulted in the fusion of exons 1 and 2 of the *TMPRSS2* gene and of exons 5 and 6 of the *ERG* gene, and the smaller variant *TMPRSS2/ERG* transcript of 350 bp resulted in the fusion of exon 1 of the *TMPRSS2* gene to exons 5 and 6 of the *ERG* gene (Figure 1*C*). Although it is conceivable that these fusion events represent independent genomic alterations occurring within one clonal tumor outgrowth, this interpretation was considered less likely given the multicentric histology of this particular tumor. The detection of these two new variant *TMPRSS2/ERG* fusions brings the total number of *ETS* gene fusions in PCa described to date to 11 (Table 1).

Applying the break-apart green (*ERG* 5′ locus) and red (*ERG* 3′ locus) FISH strategies allowed for the confirmation of *TMPRSS2/ERG* fusion in frozen sections from six different patients. Within these six patient samples, deletion between *TMPRSS2* and *ERG* was detected in three samples. In all cases, enumeration with flanking *TMPRSS2* (pale blue) and 5′ *ERG* (green) in tumors showed that the ratio was < 0.80, consistent with deletion affecting the intervening genomic DNA. Additional preliminary data have confirmed that a subset of these tumors demonstrated hemizygous deletion by oligonucleotide array-based comparative genomic hybridization (aCGH) (Human Genome CGH Microarray Kit 44B; Agilent Technologies, Inc., Palo Alto, CA) intervening in *TMPRSS2/ERG* fusion (data not shown).

**Table 1.** *ETS* Fusions Documented in PCa to Date.

| Variant Name | Exon Breakpoint | | | | Source |
|---|---|---|---|---|---|
| | *TMPRSS2* | *ERG* | *ETV1* | *ETV4* | |
| 1a | 1 | | 4 | | [3] |
| 1b | 2 | | 4 | | [3] |
| A | 1 | 4 | | | [3] |
| B | 1 | 2 | | | [3] |
| C | 2 | 5 | | | This study |
| D | 1 | 5 | | | This study |
| E | 5 | 4 | | | [5] |
| F | 4 | 5 | | | [5] |
| G | 4 | 4 | | | [5] |
| A | Pre-1 (47 bp upstream) | | | Pre-3 (19 bp) | [4] |
| B | Pre-1 (13 bp upstream) | | | Pre-3 (19 bp) | [4] |

*Genomic Architecture and Origin of Genetic Translocations*

Although there are many transcripts recognized, the human *ETV1* gene has up to 14 exons with a DNA binding domain in the last exon, whereas the *ERG* gene has 11 exons with recognized functional domains occurring across exons 5 and 6 [pointed (PNT) domain interaction] and exon 11 (*ETS* DNA binding domain). *TMPRSS2* has 14 exons with functional domains in the latter half of the protein only. Both *ERG* and *TMPRSS2* lie on chromosome 21 at cytobands 21q22.3 and 21q22.2, respectively, with approximately 3 Mb between them, and *TMPRSS2* localized more telomerically than *ERG*. As can be seen in Figure 2, the 5′ end of both genes faces the telomere. Both genes have the same transcriptional orientation and are separated by 3 Mb of DNA. Given this genomic organization and the observation that 5′ *TMPRSS2* fuses in-frame with 3′ *ERG*, interstitial deletion of the intervening 3 Mb of DNA must take place. Indeed, our three-color FISH analysis confirms the loss of genomic content from this region of chromosome 21. This finding raises the question of concomitant haploinsufficiency of one or more genes mapping to this deleted interval. Deletion of the *TMPRSS2* coding region resulting from fusion rearrangement may lead to haploinsufficiency of the gene. However, a *TMPRSS2* knockout mouse with no apparent phenotype was recently reported [7]. Examination of the 13 genes within this region of chromosome 21 (*ETS2*, *DSCR*, *BRWD1*, *HMGN1*, *C21orf13*, *SH3BGR*, *B3GALT5*, *PCP4*, *DSCAM*, *BACE2*, *FAM3*, *MX2*, and *MX1*) identified one candidate locus *HMGN1*. Knockout models of this gene demonstrated that loss increased N-cadherin expression [8] (which has been noted in high-grade PCa) [9] and altered $G_2$/M checkpoint [10]. Interestingly, COPA analysis using the Oncomine database [11] of PCa (an expression microarray dataset) [12] demonstrated that, when *ERG* was overexpressed, there was concomitant reduction in the expression of *HMGN1*.

The clarification of the exact genomic architecture will involve the sequencing or the fiber FISH analysis of samples that have been found to express the fusion transcript. Similar rearrangements involving *ETS* family members in the Ewing family of tumors and hematologic malignancies have been shown to involve classic, complex, or variant translocations. Interstitial deletions have been described in leukemias [13] and congenital syndromes, and are thought to be due to defective homologous recombination [14], which is perhaps related to DNA matrix attachment regions or areas of microhomology. In this regard, it is noteworthy that, for every case of *ERG* fusion transcript, there is at least one area of up to 300 bp on the intron following the transcribed *TMPRSS2* exon that displays microhomology with up to 90% identity to multiple areas on the intron preceding the relevant *ERG* exon of the transcript.

*Functional Implications*

The most pressing need in future work is the need to demonstrate the existence of functional protein from these transcripts. From the analysis of fusion transcripts, it is evident that *TMPRSS2* contributes an androgen-responsive regulatory region to *ERG*, *ETV1*, and *ETV4*. Identification of the contribution of other structural regions, such as the PNT domain, which is important for *TMPRSS2/ERG* and *TMPRSS2*/*ETV1* function, and elucidation of the precise functional properties of each fusion protein are areas of significant future interest. Indeed, the only other study examining *ERG* expression in PCa, using an antibody to the conserved C-terminus, found expression in 7 of 25 of high-grade cancers [15].

The *ETS* family of transcription factors encodes nuclear transcription factors with an evolutionarily conserved *ETS* domain of 85 amino acids that mediate binding to purine-rich DNA residues with more than 400 target genes in the genome that are either positively or negatively regulated by them [16]. One of the most interesting findings of the study by Tomlins et al. [3] is the suggestion that fusion genes only exist in PCa and not in the precursor lesion, high-grade prostatic intraepithelial neoplasia (HPIN). This suggests that a consequence of the expression of these gene products may be the facilitation of the cellular transitions of HPIN precursors to PCa. There is some evidence that *ETS*-related genes are involved in invasiveness and metastases [16], but these novel transcriptomes are yet to be fully characterized. Evidence to date suggests that *ERG* may be involved in histone methylation [17], inhibition of apoptosis [18], and transcriptional synergy with Jun/Fos heterodimers [19], and, when overexpressed, display transforming abilities [20]. However, potentially more relevant insights into their dysregulated mechanisms can be gained from examining their role in the Ewing family of tumors and hematologic neoplasms, where similar fusion products are known to occur. For example, both *ERG* and *ETV1* fusion products have been shown to downregulate the TGF-$\beta$2 receptor [21]—a potential tumor suppressor in PCa [22].

*Clinical Implications*

The clinical implications of translocation remain unclear. Intuitively, a translocation would appear to be detrimental; however, Petrovics et al. [2] described *ERG* overexpression in a subset of 95 PCa patients and noted that high levels were associated with a variety of different positive prognostic variables such as longer prostate-specific antigen (PSA) recurrence-free survival, well and moderately differentiated stages, lower pathological T stage, and negative surgical margins. Subsequently, the paper by Tomlins et al. [3] indicated that, in greater than 90% of cases overexpressing *ERG* or *ETV1*, a *TMPRSS2* fusion event was detectable. The possibility that the presence of a gene fusion is a positive prognostic factor raises the possibility that there may be multiple pathways toward prostatic carcinogenesis, with varying malignant potentials. In our small cohort, we did not notice any particular clinical outcome with samples carrying the rearrangement, and clinical characteristics were distributed unremarkably; tumor stages ranged from T2a to T3b—five tumors were Gleason 7 and one tumor was Gleason 9. A future area of interest for future investigation is the response to androgen deprivation in PCa. The response is known to be heterogeneous, with the median response before PSA relapse being approximately 24 months [23].

The nature of this genomic rearrangement suggests that it may be fundamental to driving carcinogenesis; thus, further hypotheses may suggest that those with an *ETS* gene fusion have a prolonged response to androgen deprivation than those without.

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