

Expression of Variant TMPRSS2/ERG Fusion Messenger RNAs Is Associated with Aggressive Prostate Cancer

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Abstract

Recent studies have reported that the majority of prostate cancers express fusion genes in which the 5' region of the androgen-regulated *TMPRSS2* gene is fused to an ETS family transcription factor, most commonly the *ERG* gene. We have characterized in detail the expression of TMPRSS2/ERG fusion mRNAs and correlated the isoforms expressed and expression levels with clinical outcome in cancers from men undergoing radical prostatectomy. Overall, 59% of clinically localized prostate cancers express the TMPRSS2/ERG fusion gene, confirming the initial observations of high frequency expression of this fusion mRNA in prostate cancer. There was significant variation in the alternatively spliced isoforms expressed in different cancers. Expression of an isoform, in which the native ATG in exon 2 of the *TMPRSS2* gene is in frame with exon 4 of the *ERG* gene, was associated with clinical and pathologic variables of aggressive disease. Expression of other isoforms, in which the native ERG ATG in exon 3 was the first in-frame ATG, was associated with seminal vesicle invasion, which is correlated with poor outcome following radical prostatectomy. Cancers not expressing these isoforms tended to express higher levels of fusion mRNAs, and in this group, higher expression levels of fusion mRNA were present in cancers with early prostate-specific antigen recurrence. Thus, both the isoforms of TMPRSS2/ERG fusions expressed and expression level may affect prostate cancer progression. (Cancer Res 2006; 66(17): 8347-51)

Introduction

Chromosomal rearrangements resulting in gene fusions with expression of functional fusion proteins are common in leukemias, lymphomas, and sarcomas (1). Such rearrangements are often linked to specific tumor phenotypes (e.g., the Philadelphia chromosome in chronic myelogenous leukemia). In contrast, gene fusions with expression of fusion proteins have generally been considered to be rare events in common epithelial malignancies, such as lung, colon, and breast cancers. The recent description by Tomlins et al. (2) of recurrent fusion of the androgen-regulated *TMPRSS2* gene to the ETS transcription factors *ERG* or *ETV1* in the majority (79%) of prostate cancer samples examined by fluorescence *in situ* hybridization challenges the general assumption that such rearrangements are rare in epithelial malignancies. At the RNA level, 20 of 42 samples examined expressed one of the fusion mRNAs, with almost all of these being ERG fusion mRNAs (19 of 20), although this sample was not random, in that it was selected

based on prior cDNA microarray or quantitative RT-PCR determinations of ERG and ETV1 expression levels. More recently, fusion of the *TMPRSS2* gene with the *ETV4* gene has been reported (3), but this seems to be considerably less common (2-3% of cases). Fusion proteins involving ETS transcription factors are characteristic of Ewing's tumors and occur at lower rates in some leukemias (4). Based on this finding and the known biology of these transcription factors and fusion proteins, these genes are considered as oncogenes that promote neoplastic progression.

ERG has been identified previously as a gene that was commonly overexpressed in prostate cancer by Petrovics et al. (5). These authors showed increased ERG mRNA in 62% of prostate cancers using quantitative reverse transcription-PCR (RT-PCR) primers from the 3' portion of the *ERG* gene, distal to the region involved by the TMPRSS2 fusion. Paradoxically, they observed an inverse correlation of ERG gene expression and prostate cancer aggressiveness. This is unexpected given the biology of the ETS transcription factors and the high rate of gene fusion with *TMPRSS2* in prostate cancer. Although it is possible that in some cases ERG is being overexpressed due to other genetic or epigenetic changes in prostate cancer, this seems to be uncommon because Tomlins et al. (2) found that 95% of cases with ERG overexpression also had expression of the TMPRSS2/ERG fusion gene. To elucidate the role of the TMPRSS2/ERG fusion gene in prostate cancer, we have examined in detail the expression of the TMPRSS2/ERG fusion mRNAs in prostate cancers with early, late, or no recurrence after 5 years of follow-up. We have found marked heterogeneity in the expressed forms of the TMPRSS2/ERG fusion mRNA. Expression of a fusion mRNA with the native TMPRSS2 translation initiation site in frame with the *ERG* gene is associated with early biochemical recurrence following radical prostatectomy. Such early recurrence is a hallmark of aggressive disease and is significantly associated with cancer progression and death from disease (6). Other variants with the native ERG translation initiation codon as the first in-frame ATG are associated with seminal vesicle invasion, a pathologic hallmark of aggressive prostate cancer. Thus, the type of ERG fusion mRNA expressed is an important determinant of disease outcome in cancers with the TMPRSS2/ERG gene fusion. Cancers that do not express these isoforms tended to have higher overall expression of fusion mRNA perhaps as a compensation for lower potency fusion mRNAs without native ATGs as the first in-frame translation initiation codon. Thus, both the isoforms of TMPRSS2/ERG fusions expressed and expression level may affect prostate cancer progression.

Materials and Methods

Patients and cell line cDNAs. All radical prostatectomy tissue samples were obtained from Baylor Prostate Specialized Programs of Research Excellence (SPORE) Tissue Core and collected from fresh radical prostatectomy specimens after obtaining informed consent under an institutional review board-approved protocol. RNAs were prepared from

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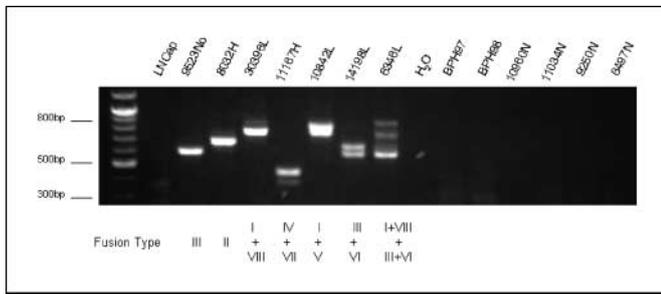


Figure 1. RT-PCR amplification of TMPRSS2/ERG fusion transcripts in prostate cancer. Lanes 2 to 8, cancer samples; lanes 10 and 11, two BPH samples; lanes 13 to 16, normal prostate. LNCaP cell line (lane 1) and H₂O (lane 9) are negative controls. Samples 9523No and 8032H are representative for single fusion RT-PCR products type III or II; the other cancer samples represent different combinations of multiple fusion RT-PCR products.

prostate cancer tissues or benign tissues as described previously (7). There were 19 normal samples (peripheral zone tissue), 18 early recurrence cancers [prostate-specific antigen (PSA) recurrence in <1 year], 16 late recurrence cancers (PSA recurrence after 1-5 years), 20 nonrecurrent cancers (no PSA recurrence in 5 years), 5 cases with at least 16 months follow-up with no PSA recurrence but who were lost to follow-up before 5 years, and 8 benign prostate hyperplasia (BPH) tissues. PSA recurrence is defined as having two successive follow-up PSA values >0.2 ng/mL >30 days following surgery. Cancer samples were at least 70% cancer. The LNCaP cell line, reported to be TMP/ERG fusion negative (2), was maintained and RNA was extracted as described previously (8). Each RNA sample (5 µg) was reversed transcribed into cDNA as described previously (7).

RT-PCR and quantitative real-time PCR. Primers for TMP/ERG fusion detection were TMPRSS2 RT forward 5'-CAGGAGCGGAGCGCGA-3' and TMPRSS2:ERG RT reverse 5'-GCGGTTGTAGCTGGGGTGAG-3'. RT-PCR amplifications were done using 5 µL cDNA as template in a final volume of 25 µL according to the instruction of Takaza PCR kit (Takaza Bio, Inc.,

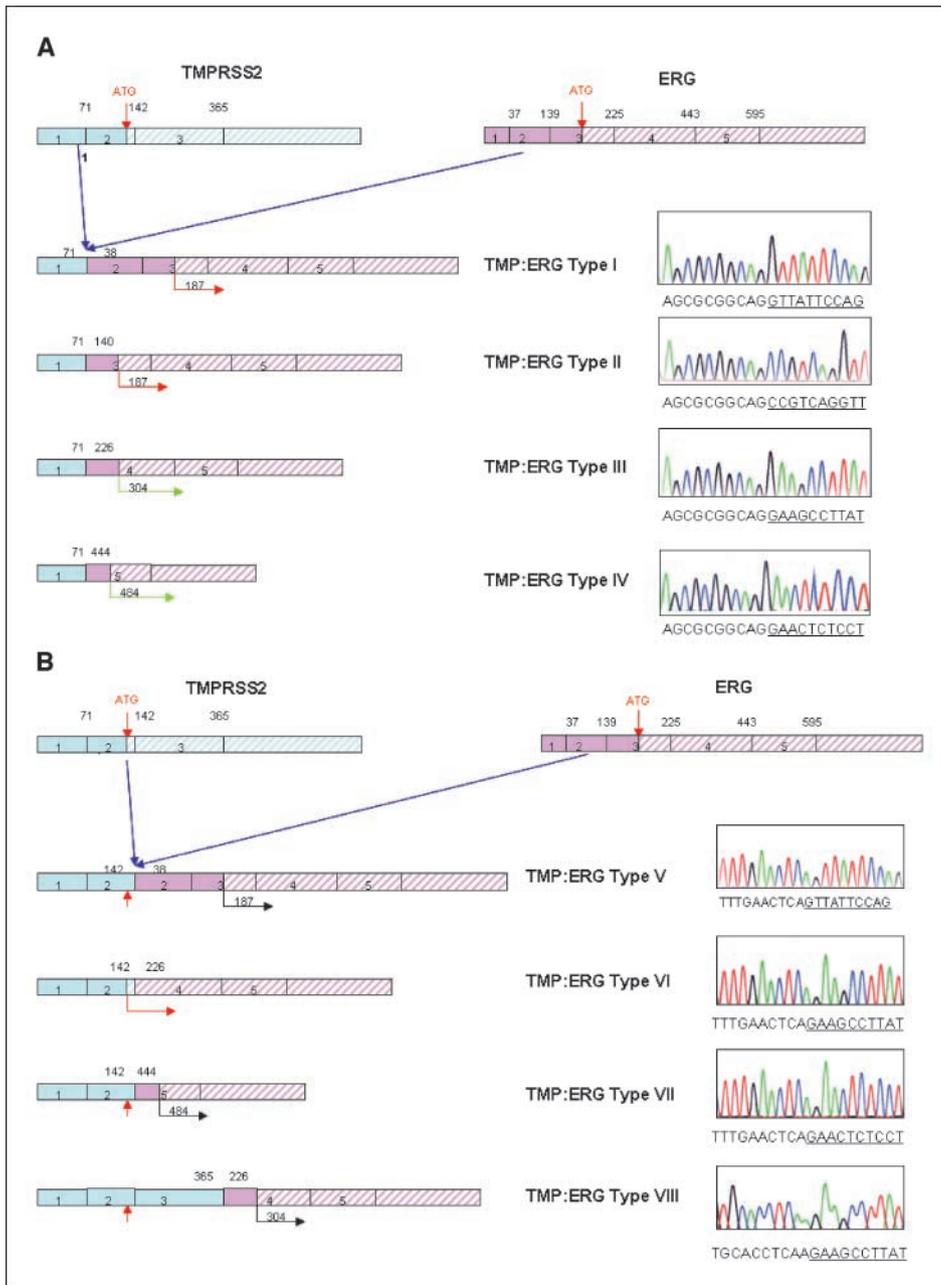


Figure 2. Identification and characterization of TMPRSS2/ERG fusions mRNAs in prostate cancer. A, schematic structure of TMPRSS2/ERG fusion mRNAs types I to IV. Exons (boxes) for TMPRSS2 (blue) and ERG (purple) are numbered according to Genbank reference sequences (NM_005656 and NM_004449). Numbers above the exons, last base of each exon; vertical red arrow, native ATG; hatch marks, longest open reading frame. For each type of fusion, numbers above the fusion site are the last base pair in TMPRSS2 and the first base pair in ERG gene. Insets, position and automated DNA sequencing of the fusion sites, with the ERG sequence underlined. Horizontal arrow, first in-frame translation initiation site; its location in the ERG gene is shown. Red, translations from native ATGs; green, translations from nonnative ATGs. B, schematic structure of TMPRSS2/ERG fusion mRNAs types V to VIII. Fusion mRNAs are illustrated as described above. Red vertical arrows, position of the out-of-frame native TMPRSS2 ATG; black horizontal arrow, first in-frame ATG in the ERG gene; horizontal red arrow, TMPRSS2/ERG fusion protein initiation site in the type VI mRNA.

Table 1. Summary of *TMPRSS2/ERG* fusion types

Types	TMP	ERG	Size	Description of TMP:ERG fusion	n (%)
I	12-71	38-762	779	Exon 1 of TMP + beginning of ERG exon 2	7 (20)
II	12-71	140-762	678	Exon 1 of TMP + beginning of ERG exon 3	4 (11)
III	12-71	226-762	591	Exon 1 of TMP + beginning of ERG exon 4	30 (86)
IV	12-71	444-762	373	Exon 1 of TMP + beginning of ERG exon 5	1 (3)
V	12-142	38-762	850	Exons 1 and 2 of TMP + beginning of ERG exon 2	3 (9)
VI	12-142	226-762	662	Exons 1 and 2 of TMP + beginning of ERG exon 4	9 (26)
VII	12-142	444-762	444	Exons 1 and 2 of TMP + beginning of ERG exon 5	2 (6)
VIII	12-365	226-762	885	Exons 1-3 of TMP + beginning of ERG exon 4	2 (6)

NOTE: Fusion sites, locations within each gene, lengths of fusion PCR products (in bp), and number and percentage of cancers expressing each type of fusion. Base pair numbers are based on Genbank accession nos. NM_005656 (*TMPRSS2*) and NM_004449 (*ERG*). Percent is expressed as the percentage with that isoform of all cancers expressing any fusion mRNA.

Shiga, Japan). The PCR was done using a standard three-step protocol with annealing temperature of 65°C. Amplified PCR fragments were run on a 1.2% agarose gel and stained with ethidium bromide. Different sized PCR products were cloned into PCR 2.1-Topo vector from Topo TA Cloning kit (Invitrogen, Carlsbad, CA). Plasmids were prepared by using the Qiagen Spin Mini-Prep kit (Qiagen, Inc., Valencia, CA) and then sequenced by M13R and M13F primers. Sequence analysis for *TMPRSS2* (NM_005656) and *ERG* (NM_004449) was done by using National Center for Biotechnology Information BLAST software.¹ The same primer set was used in for quantitative real-time PCR. β -Actin primers were as described previously (8). Template cDNA or standard control vectors (5 μ L), which contained the *TMPRSS2/ERG* fusion type III or β -actin fragment, were used in a final reaction volume of 25 μ L. The standard control vectors were prepared into copy number and a dilution series of each plasmid from 10⁸ to 10² was used as DNA standard for real-time PCR. The Master Mix for real-time PCR contained 2 mmol/L MgCl₂, 0.4 μ mol/L each of forward and reverse primers, and 2.5 μ L LC-FastStart DNA Master SYBR Green (10 \times ; Roche, Indianapolis, IN). Real-time PCR was done by using the iCycler iQ instrument from Bio-Rad (Hercules, CA) followed by a two-step PCR protocol with annealing temperature of 68°C. All real-time PCR efficiencies were controlled in the range of 100 \pm 5%.

Results and Discussion

To determine the frequency of expression of the *TMPRSS2/ERG* fusion gene in clinically localized cancers, we analyzed RNAs from 59 clinically localized cancers using RT-PCR followed by agarose gel electrophoresis. The cases included 18 cancers with early PSA recurrence cancers (<1 year), 16 cancers with late recurrence (PSA recurrence between 1 and 5 years), 20 nonrecurrent cancers (no PSA recurrence with minimum of 5 years), and 5 cancers without early recurrence but without 5 years follow-up. There were 35 *TMPRSS2/ERG* fusion mRNA-positive samples found in our 59 cancer samples (59%). The proportion of positive samples did not differ significantly between recurrence groups (12 of 18 early, 10 of 16 late, and 12 of 20 nonrecurrent). Our results are very similar to those reported by Tomlins et al. (2), who found expression of the *TMPRSS2/ERG* fusion gene in 15 of 32 (47%) clinically localized cancers. Recently, Soller et al. (9) have reported expression of the *TMPRSS2/ERG* fusion gene in 14 of 18 prostate cancers (78%), although the clinical stage of these cancers was not reported. Thus, expression of the *TMPRSS2/ERG* fusion gene is present in the

majority of prostate cancers. We found no expression of the *TMPRSS2/ERG* fusion in 28 benign prostate samples (20 normal peripheral zone and 8 hyperplastic transition zone), confirming the specificity of this fusion for cancer tissues.

During this analysis, we noted very significant heterogeneity in the size of the PCR products obtained, with many samples having several bands on agarose gel electrophoresis (Fig. 1). To confirm whether these represent actual fusion gene transcripts and, if so, to analyze their structure, we cloned and sequenced the *TMPRSS2/ERG* RT-PCR products. There were in total eight different sizes of PCR products identified ranging from 373 to 885 bp. The results of the sequence analysis are summarized in Fig. 2 and Table 1. The *TMPRSS2/ERG* fusion mRNAs can be divided into two broad groups. As shown in Fig. 2A, types I to IV fusions all contain the first exon of *TMPRSS2* (1-71 bp), which is juxtaposed to exon 2, 3, 4, or 5 of *ERG*. Types I and II can initiate translation from the native *ERG* translation initiation codon in exon 3 of the *ERG* gene, whereas types III and IV would have to initiate translation from an internal ATG because exon 1 of *TMPRSS2* is noncoding and does not contain an ATG (Fig. 2A). The proteins encoded by types III and IV mRNAs would be predicted to be 39 and 99 amino acids shorter, respectively, than full-length *ERG* if translation is initiated from the first in-frame ATG. We compared our finding with two reported fusion types of Tomlins et al. (2). Their TMP:ERGb is the same as our type I, whereas their TMP:ERGa is the same as our type III. As shown in Fig. 2B, types V to VIII all have the first two or three exons of *TMPRSS2* (1-142 or 1-365 bp), which includes the *TMPRSS2* translation initiation ATG codon in exon 2. Of note is the fact that the *TMPRSS2* ATG in the type VI fusion is in frame with the *ERG* protein starting in exon 4. It would include the first 5 amino acids of *TMPRSS2* but would lack the first 12 amino acids of the full-length *ERG* protein. For types V, VII, and VIII, the *ERG* protein is out of frame with the *TMPRSS2* reading frame initiating at the *TMPRSS2* ATG; therefore, to yield functional protein, these would need to initiate translation from internal ATGs within the *ERG* portion of the fusion gene. Of note, Soller et al. (9) detected types I, III, and VI as well as variants with *TMPRSS2* exons 4 and 5, which we did not detect. Expression of multiple fusion mRNAs was very common; overall, 17 of 35 cases that expressed fusion mRNAs had two or more products (Fig. 3A). By far the most common single fusion mRNA was type III and 86% (30 of 35) of cases expressed this type either alone or in combination with other types. The most likely explanation for the multiple forms of fusion mRNAs

¹ <http://www.ncbi.nlm.nih.gov/blast>.

is alternative splicing because it seems highly unlikely that multiple different gene fusions are present in a single cancer. Indeed, alternative splicing of the *ERG* gene has been described previously (10). Whether the differences in expression of different fusion mRNAs reflect differences in the structure of the fusion gene, differences in the activity of the splicing machinery within the cancer cells, or both is a complex question that requires further investigation.

We next sought to determine if differences in expression of different TMPRSS2/ERG fusion mRNAs were associated with clinical and pathologic variables correlated with aggressive prostate cancer. Results of this analysis are summarized in Fig. 3A. Expression of type VI fusion mRNA, in which the TMPRSS2 ATG is in frame with exon 4 of ERG, is strongly associated with aggressive disease. Six of 9 cancers with this variant had early PSA recurrences versus 6 early recurrences in 26 cancers without this type ($P = 0.038$, Fisher's exact test). There was also a statistically significant association with seminal vesicle invasion (6 of 9 versus 6 of 26; $P = 0.038$, Fisher's exact test), which is well established as pathologic variable associated with aggressive disease (11). There was no significant correlation with pelvic lymph node metastasis at the time of surgery ($P = 0.081$, Fisher's exact test), pathologic evidence of extracapsular extension, or Gleason score. We next compared outcomes for patients with types I and/or II fusion mRNAs (but without type VI), because types I and II contain the native ERG ATG from exon 3 as the first in-frame ATG, with cases without expression of type I, II, or VI fusion mRNAs. Expression of one or both of these isoforms was associated with seminal vesicle invasion ($P = 0.02$, Fisher's exact test). If case 5, which also expresses type VI fusion mRNA, this association is still significant ($P = 0.05$). There was no statistically significant association with early recurrence, lymph node metastasis, seminal vesicle invasion, extracapsular extension, or Gleason score. Expression of either type I, II, or VI fusion mRNA (17 of 35 cases) versus none of these types (18 cases) was associated with early recurrence ($P = 0.035$) and seminal vesicle invasion ($P = 0.005$). Thus, expression of fusion mRNAs containing the native translation initiation codons in frame with the ERG protein, particularly the TMPRSS2 ATG (type VI), is associated with aggressive disease.

The reason for the association of expression of the type VI and, to a lesser extent, types I and II isoforms with aggressive disease is not known. We favor the hypothesis that these genes are translated more efficiently from the native translation initiation codons compared with internal ATGs. Regulation of site of translation initiation and the efficiency of this process is complex (for recent review, see ref. 12). However, it should be noted that the TMPRSS2 ATG has an ideal Kozak consensus sequence, with an A at -3 and G at $+4$ (12, 13). The native ERG ATG in exon 3, which is the initial in-frame ATG in types I and II, is less ideal, with a G at -3 and A at $+4$ (12). For the most common isoform (type III), the first in-frame ATG (in exon 4) has the same sequence at positions -3 and $+4$ and does not have a C at -1 , which is thus far the most common nucleotide in this position in native initiation codons (13). In addition, based on the scanning theory of translation initiation (12), the ideal TMPRSS2 ATGs that are out of frame with the ERG protein (types V, VII, and VIII) should substantially inhibit production of ERG protein. As an alternative explanation, it is also possible that the unique amino acid sequence at the amino terminus of type VI fusion protein alters its biological properties. Finally, it is possible that the expression of the types I, II, and VI variants is simply a reflection of altered splicing activity in the cancer cells and it is this alteration that is driving, or is associated

with, tumor progression. Clearly, further comprehensive investigations are needed to answer this question.

We next examined the expression level of the TMPRSS2/ERG fusion mRNAs by quantitative RT-PCR using a primer set that detects all isoforms. There was a very large range of expression of fusion mRNAs from <100 to 24,065 copies/ 10^6 β -actin transcripts (Fig. 3B). Several observations are worth noting. First, the mean expression level in cases expressing the either type I, II, or VI mRNAs was lower (2,485 copies/ 10^6 β -actin transcripts) than cases

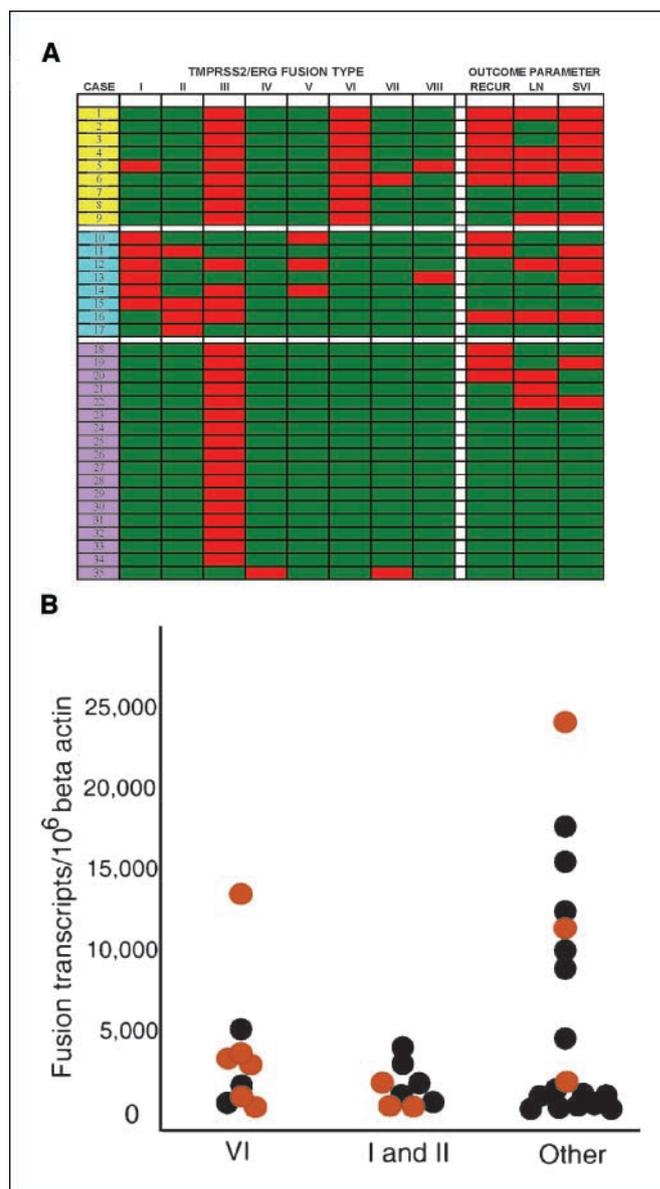


Figure 3. Association of TMPRSS2/ERG fusion isoform expression with clinical and pathologic variables in cancers from radical prostatectomy specimens.

A, presence (red) and absence (green) of a fusion protein isoform or clinical/pathologic variable. TMPRSS2/ERG fusion types are as shown in Fig. 2 and Table 1. Yellow, cases that express the type VI isoform; blue, cases that express types I and/or II isoforms but not type VI; purple, cases that do not express type I, II, or VI isoforms. RECUR, early PSA recurrence; LN, pelvic lymph node metastasis; SVI, seminal vesicle invasion. **B**, expression of TMPRSS2/ERG fusion transcripts was determined by quantitative RT-PCR and normalized to β -actin copy number. Red, cases with early PSA recurrence. VI, all cases with expression of type VI isoforms; I and II, cases with type I and/or II isoforms but no type VI isoforms; Other, cases not in the first two groups, primarily cases expressing only the type III isoform.

not expressing any of these isoforms (6,145 copies/ 10^6 β -actin transcripts). As shown in Fig. 3B, there were many more cases with high expression of *TMPRSS2/ERG* fusion mRNA among those cases that did not express type I, II, or VI isoforms (Fig. 3B). For cases expressing type I, II, or VI isoforms, the mean expression ± 2 SDs above the mean was 8,731 copies/ 10^6 β -actin transcripts. Only 1 of 17 such cases exceeded this expression level. In contrast, 7 of 18 cases that did not express type I, II, or VI isoforms had $>8,731$ copies/ 10^6 β -actin transcripts. This difference is statistically significant ($P = 0.041$, Fisher's exact test). Thus, although expression levels are quite variable, cases without type I, II, or VI isoforms tended to have higher expression levels as might be expected if the isoforms expressed are less active biologically per transcript. These observations may account for the paradoxical observation of Petrovics et al. (5) of an inverse correlation of *ERG* gene expression and prostate cancer aggressiveness, because the presumably more biologically potent isoforms that are associated with aggressive disease tend to be expressed at lower levels, whereas cancers that do not express these isoforms tend to express overall higher levels of *ERG* mRNA perhaps due to selection for higher expression. Supporting this concept is the observation that among the cases without type I, II, or VI transcripts, the mean expression levels in cancers with early recurrence was 12,443 copies/ 10^6 β -actin transcripts, whereas those without early recurrence had a mean expression level was 4,886 copies/ 10^6 β -actin transcripts. Thus, both overall expression levels and the particular fusion isoforms expressed may affect disease progression.

The studies described above confirm that the presence of the *TMPRSS2/ERG* fusion gene is very common in prostate cancer. Furthermore, our data are consistent with the idea that both the isoforms of the fusion mRNA expressed and their expression level may affect disease progression. Additional studies of large independent series of prostate cancers are needed to confirm this concept. In addition, studies correlating mRNA isoforms and expression levels and fusion protein levels in prostate cancer tissues are needed. Finally, further mechanistic studies of translation initiation and examination of the activities of the

different isoforms are needed to understand the biological basis of the association observed in clinical samples.

The *TMPRSS2* gene encodes a transmembrane-bound serine protease that is highly localized to prostate and is overexpressed in neoplastic prostate epithelium (14, 15). Nelson's group reported that a 15-bp sequence located at *TMPRSS2* promoter region is homologous to the consensus androgen response element, which is in accordance with androgen-inducible expression of the gene (14) as well as experiments suggesting that the androgen-responsive promoter elements of *TMPRSS2* mediate the overexpression of ETS family members in prostate cancer (2). Our group, in collaboration with others (16), has shown that increased expression of the steroid receptor coactivator-1, which acts via the androgen receptor, is associated with aggressive prostate cancer. The *TMPRSS2/ERG* fusion is an obvious androgen receptor target gene that may mediate prostate cancer aggressiveness. Further investigations of the transcriptional control of the *TMPRSS2/ERG* fusion gene are clearly warranted.

In our study, we found no evidence of *TMPRSS2/ERG* gene fusion in benign prostate tissue and that expression of specific fusion mRNAs is associated with aggressive disease. A diagnostic test to detect the *TMPRSS2/ERG* fusion genes in cancer cells in blood or urine should be specific for prostate cancer and detection of specific isoforms may have prognostic utility to assist in treatment planning. Further studies and development of appropriate technologies are clearly indicated. Finally, the existence of a recurrent chromosomal rearrangement in prostate cancer is a paradigm shift in the study of epithelial malignancies and other epithelial tumors, such as breast, lung, and colon cancers, may have their own recurrent chromosomal rearrangements that are yet to be identified.

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