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Comprehensive assessment of *TMPRSS2* and *ETS* family gene aberrations in clinically localized prostate cancer

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Novel recurrent gene fusions between the androgen-regulated gene *TMPRSS2* and the *ETS* family members *ERG, ETV1,* or *ETV4* have been recently identified as a common molecular event in prostate cancer development. We comprehensively analyzed the frequency and risk of disease progression for the *TMPRSS2* and *ETS* family genes rearrangements in a cohort of 96 American men surgically treated for clinically localized prostate cancer. Using three break apart (*TMPRSS2, ERG, ETV4*) and one fusion (*TMPRSS:ETV1*) fluorescence *in situ* hybridization (FISH) assays, we identified rearrangements in *TMPRSS2*, *ERG*, *ETV1*, and *ETV4* in 65, 55, 2, and 2% of cases, respectively. Overall, 54 and 2% of cases demonstrated *TMPRSS2:ERG* and *TMPRSS2:ETV1* fusions, respectively. As intronic loss of genomic DNA between *TMPRSS2* and *ERG* has been identified as a mechanism of *TMPRSS2:ERG* fusion, our assays allowed us to detect deletion of the 3′ end of *TMPRSS2* and the 5′ end of *ERG* in 41 and 39% of cases rearranged for respective genes. Prostate cancers demonstrating *TMPRSS2* gene rearrangement were associated with high pathologic stage ($P = 0.04$). Our results confirm that recurrent chromosomal aberrations in *TMPRSS2* and/or *ETS* family members are found in about 70% of prostate cancers. Importantly, we define a novel approach to study these gene fusions and identified cases where *TMPRSS2* was rearranged without rearrangement of *ERG*, *ETV1* or *ETV4* and cases with *ETS* family gene rearrangement without *TMPRSS2* rearrangement, suggesting that novel 5′ and 3′ partners may be involved in gene fusions in prostate cancer.
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Despite being one of the most prevalent cancers and a major leading cause of morbidity and mortality, crucial events in prostate cancer development remain unclear. Recently, by applying a new bioinformatics approach, our group identified and validated novel recurrent gene rearrangements in majority of prostate cancers fusing the 5′-untranslated region of androgen-regulated gene *TMPRSS2* (21q22.3) with the *ETS*-transcription factor family members, *ERG* (21q22.2), *ETV1* (7q21.2), or *ETV4* (17q21).[1,2] We also observed early on that the majority of *TMPRSS2:ERG* gene fusions prostate cancers were associated with a heterogeneous intronic deletion between *TMPRSS2* and *ERG* on chromosome 21q22.2–3 as determined by both fluorescence *in situ* hybridization (FISH) and single nucleotide polymorphism array analysis.[3] This was independently confirmed by Yoshimoto *et al*.[4]

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Subsequently, Perner *et al*[5] described *TMPRSS2:ERG* fusion in 19% of high-grade prostatic intraepithelial neoplasia lesions present adjacent to cancer foci, suggesting it as an early molecular event associated with invasion. The identification of recurrent gene fusions in prostate cancer has defined a new paradigm for understanding the biology of prostate cancer development.

With the widespread use of serum prostate-specific antigen (PSA) screening, over 90% of the prostate cancers diagnosed in American men are clinically localized with 100% 5-year survival.[6] Whether these clinically localized cancers should be treated, and if, treated how aggressively remains an important management dilemma.[7,8] Currently, the clinical stage, biopsy Gleason grade and serum PSA levels are used for prognostication and treatment stratification at the time of diagnosis,[9] however, these indicators do not always accurately predict clinical outcome on an individual patient basis. The identification of the common *TMPRSS2:ETS* gene fusions in prostate cancer suggests, that distinctive molecular subtypes may define the risk of disease progression. In addition, yet uncharacterized *TMPRSS2:ETS* fusions or *TMPRSS2:ETS* fusion negative cancers harboring unique gene fusions may exist and represent additional molecular subtype. A recent study by Demichelis *et al*[10] also indicated that there are potentially important differences in the frequency of these gene fusions between population-based *vs* a hospital-based patient cohort. In the current study, we comprehensively analyzed the *TMPRSS2* and *ETS* family genes rearrangement status using three break apart (*TMPRSS2, ERG,* and *ETV4*) and one fusion (*TMPRSS2:ETV1*) FISH assay in a nonpopulation-based cohort of American men surgically treated for clinically localized disease.

Materials and methods

Study Population, Clinical Data, and Prostate Sample Collection

A tissue microarray (TMA) containing 360 cores representing clinically localized prostate cancers and benign tissue was constructed from 96 men who underwent radical prostatectomy at the University of Michigan as the primary monotherapy (ie, no adjuvant, or neoadjuvant, hormonal or radiation therapy). This radical prostatectomy series is part of the University of Michigan Prostate Cancer Specialized Program of Research Excellence (SPORE) Tissue Core. All patients provided written informed consent, and this study was approved by the Institutional Review Board at the University of Michigan Medical School. Three cores (0.6 mm in diameter) were taken from each representative tissue block to construct the TMA as described.[11,12] Detailed clinical, pathological, and TMA data are maintained on a secure relational database as described previously.[13]

Assessment of *TMPRSS2:ETS*-Gene Fusion Using an Interphase FISH Assay

About 4 μm thick TMA sections were used for interphase FISH, processed, and hybridized as described previously.[1,2] Slides were examined using an Axioplan ImagingZ1 microscope (Carl Zeiss) and imaged with a CCD (charged couple device) camera using the ISIS software system in Metafer image analysis system (Meta Systems, Altlussheim, Germany). FISH signals were scored manually ($\times 100$ oil immersion) by pathologists (RM and RBS) in morphologically intact and nonoverlapping nuclei and a minimum of 50 cancer cells or the maximum numbers of cancer cells available in three cores from a case were recorded. Cases without 50 evaluable cancer cells were reported as insufficient. Core with very weak signals or lack of signals was recorded as insufficient for hybridization. Cases lacking tumor tissue in all three cores were also excluded. All bacterial artificial chromosomes (BACs) were obtained from the BACPAC Resource Center (Oakland, CA, USA), and probe locations were verified by hybridization to metaphase spreads of normal peripheral lymphocytes. For detection of *TMPRSS2*, *ERG*, and *ETV4* rearrangements we used the following probes: RP11-35C4 (5′ to *TMPRSS2*) and RP11-120C17 (3′ to *TMPRSS2*), RP11-95I21 (5′ to *ERG*) and RP11-476D17 (3′ to *ERG*), and RP11-100E5 (5′ to *ETV4*) and RP11-436J4 (3′ to *ETV4*). For detection of *TMPSS2–ETV1* fusion, RP11-35C4 (5′ to *TMPRSS2*) was used with RP11-124L22 (3′ to *ETV1*). BAC DNA was isolated using a QIAFilter Maxi Prep kit (Qiagen, Valencia, CA, USA), and probes were synthesized using digoxigenin- or biotin-nick translation mixes (Roche Applied Science, Indianapolis, IN, USA). The digoxigenin-and biotin-labeled probes were detected using fluorescein-conjugated antidigoxigenin antibodies (Roche Applied Science) and Alexa 594-conjugated streptavidin (Invitrogen, Carlsbad, CA, USA), respectively.

Statistical Analysis

Statistical analyses were carried out using SAS (SAS Institute Inc., Cary, NC, USA) software. The *P*-values to test the associations between *TMPRSS2* and *ERG* fusion/deletion status and clinicopathologic features were calculated under a χ^2 test for 2×2 table and Wilcoxon rank-sum test for continuous outcomes. Event time is calculated from the date of surgery to the time of PSA failure. Patients not experiencing failure events were censored on their last date of follow-up. Probability of PSA recurrence-free survival was then calculated using the product-limit method of Kaplan–Meier. A log-rank test was used to compare the survival curves by gene

fusion status. Furthermore, Cox proportional hazards model was used to compute the hazard rate and the associated confidence interval for gene fusion status and each of the clinical parameters. Wald's test was used to determine the statistical significance in the Cox models.

Results

As the androgen-regulated gene *TMPRSS2* is the only known 5′ partner of *ETS*-family genes in all characterized cases, we employed a *TMPRSS2* split probe FISH assay approach to detect the overall frequency of gene rearrangements in prostate cancers. In a second step, we also used split probe assays for *ERG* and *ETV4* and a fusion probe assay for *ETV1* to detect the so far known *ETS*-family members as fusion partners with *TMPRSS2*. Normal signal patterns for *TMPRSS2*, *ERG,* and *ETV4* in 4′,6-diamidino-2-phenylindole-stained nuclei were indicated by two pairs of colocalized green and red signals (according to Figure 1). For these probes, a rearrangement was confirmed by break apart of one of the two colocalized signals. For *TMPRSS2–ETV1* fusion, two pairs of separate red and green were recorded as normal, whereas one pair of separate and one pair of colocalized signals was recorded as a rearrangement (Figure 1).

Of the 96 cases, 75 cases overall qualified for the assessment as described in the methods, TMPRSS2 was evaluable in 57, ERG in 65, ETV1 in 53, and ETV4 in 58 cases. Overall, *TMPRSS2* was rearranged in 65% (37/57) of cases, *ERG* in 55% (36/65), *ETV1* in 2% (1/53), and *ETV4* in 2% (1/58) of cases. Approximately half (54%) (30/56) of localized prostate cancers harbored *TMPRSS2:ERG* rearrangements (indicated by rearrangement of both *TMPRSS2* and *ERG*). *TMPRSS2* fusions with the other *ETS* partners were rare in this cohort, with one of 53 cases having a *TMPRSS2:ETV1* fusion (2%) and no cases having *TMPRSS2:ETV4* fusion. Loss of red signal corresponding to a deletion of the 3′ end of *TMPRSS2* and loss of green signal corresponding to a deletion of the 5′ end of *ERG* was identified in 41% (15/37) and 39% (14/36) of cases with rearrangements in the respective genes. The frequency and distribution of gene aberrations and deletion is summarized in Figure 2a and c. In 11% (6/57) cases *TMPRSS2* was rearranged without rearrangement in *ERG*, *ETV1*, or *ETV4*. In addition, 1/65 (2%) and 1/58 (2%) cases were rearranged for *ERG* and *ETV4* without rearrangement with *TMPRSS2*. These discordant cases are summarized in Figure 2b.

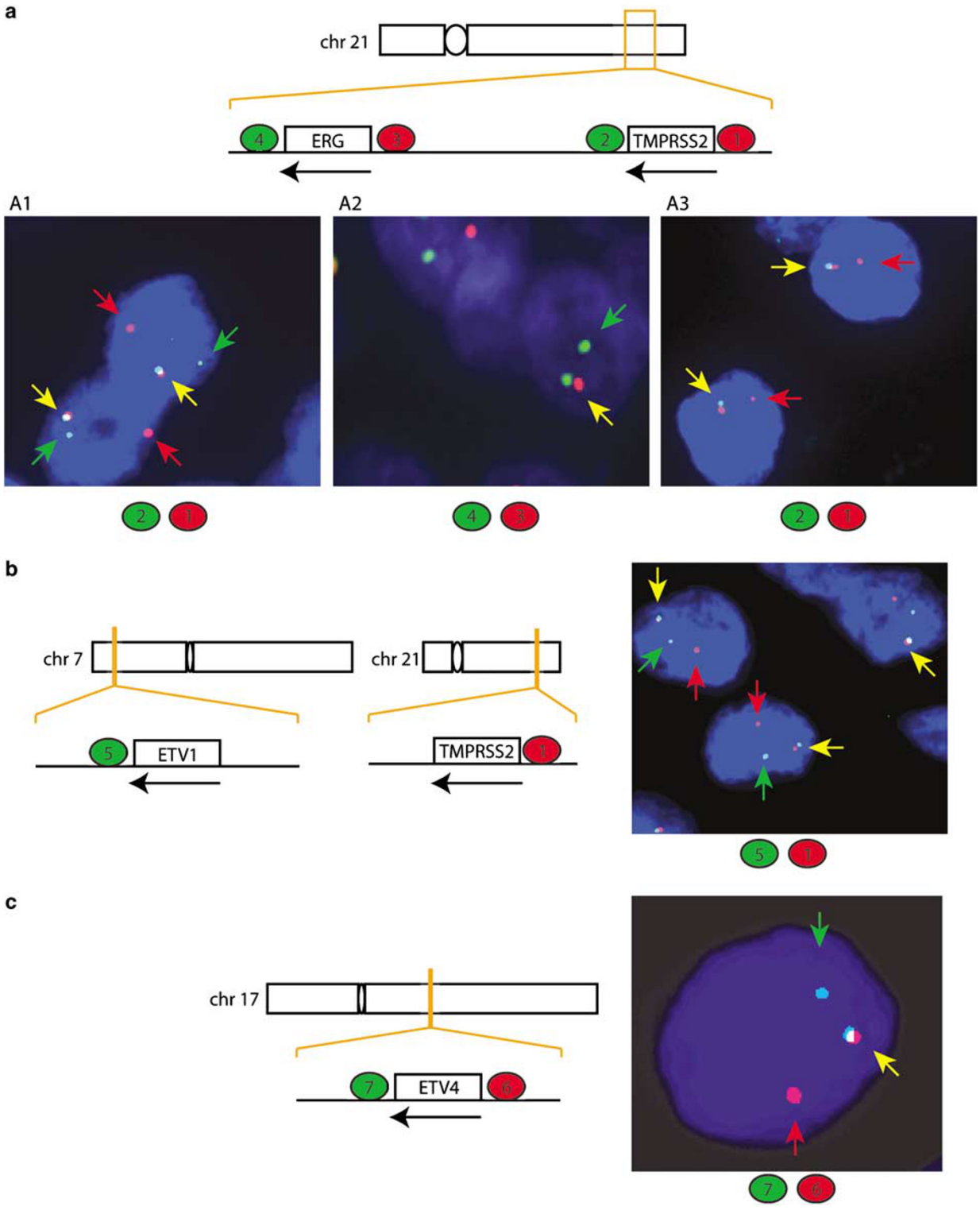
The clinical and pathological characteristics of 96 prostate cancer cases treated by radical prostatectomy are summarized in Table 1. The median post-surgery follow-up was 102.6 months (range = 2.7–124.6 months) and the average age at surgery was 61 years (range = 43–76 years). Seventy-six percent of tumors were organ confined (stage pT2), 19% of tumors had signs of local invasion (pT3a), and 5% had seminal vesicle invasion (pT3b). No cases had pelvic lymph node involvement. Among 96 patients, 34 had biochemical treatment failure defined by a post-operative PSA > 0.2 ng/ml. We explored the associations between rearrangement status and clinical and pathological variables. Prostate cancer cases with *TMPRSS2* and/or *ERG* gene rearrangement associated with or without deletions were not associated with risk of biochemical failure however there was a statistically significant association for *TMPRSS2* gene rearrangement and high pathologic stage ($P = 0.04$) (Table 2). The clinicopathological associations of prostate cancer cases associated with *TMPRSS2* and *ERG* gene rearrangement is summarized in Table 2.

Discussion

This is the first report to evaluate overall frequency of gene aberrations in a hospital-based cohort of American men treated for clinically localized prostate cancer. For this purpose we employed either split probe or fusion probe approach for all of the known *TMPRSS2* and *ETS*-fusion partners (ie *ERG, ETV1,* and *ETV4*). Our results demonstrate the complex rearrangement status of *TMPRSS2* and *ETS* family genes in clinically localized prostate cancer (Figures 2a–c). The 54% frequency of *TMPRSS2:ERG* gene fusions in this cohort is comparable to the 55% (16/29) reported in our original discovery,[1] 49% (58/118) recently reported by Perner *et al*,[3] 78% (14/18) by Soller *et al*[14] and 40% (6/15) by Yoshimoto *et al*.[4] The frequency of *ETV1* gene fusion in the current study is rare (2%)

Figure 1 Assays approach to detect *TMPRSS2:ETS* gene fusions in prostate cancer. Schematic and representative positive results from four assays employing interphase FISH on formalin-fixed, paraffin-embedded tissues to detect *TMPRSS2:ETS* gene fusions in prostate cancer. For all assays, the chromosomal location of the gene is indicated (boxes), with the direction of transcription indicated by the arrow. 5′ and 3′ BACs are indicated in ovals, with the number identifying the BAC as described below and the color indicating the probe color in the accompanying images. Green and red arrows show individual signals, whereas yellow arrows indicate colocalized signals. (**a**) A1, *TMPRSS2* rearrangement positive case (without deletion), as indicated by one pair of split 5′ and 3′ signals. A2, An *ERG* rearrangement positive (with deletion) prostate cancer case showing loss of one red labeled probe 5′ to *ERG*. A3, A *TMPRSS2* rearrangement positive (with deletion) prostate cancer case showing loss of one green-labeled probe 3′ to *TMPRSS2*. (**b**) Fusion assay for *TMPRSS2:ETV1* gene fusions. A *TMPRSS2:ETV1* fusion positive case is shown, as indicated by one pair of fused 5′ *TMPRSS2* and 3′ *ETV1* signals. (**c**) Break apart assay for *ETV4*. Prostate cancer cells showing a rearrangement of *ETV4* as indicated by break apart of the yellow signal (yellow arrow) of one allele to generate distinct separate 5′ and 3′ probes (red and green arrows). BACs are as follows: 1 = RP11-35C4, 2 = RP11-120C17, 3 = RP11-95I21, 4 = RP11-476D17, 5 = RP11-124L22, 6 = RP11-100E5, and 7 = RP11-436J4.

compared to our initial report of 31% (7/23). However, our initial report describing the frequency of these novel gene fusions was rather based on selected prostate cancer samples from both clinically localized and metastatic prostate cancers. Perner *et al*[3] did not observe any examples of *TMPRSS2:ETV1* gene fusions in a total of 30 cases of prostate cancer, xenografts, or cell lines. Two recent studies by Yoshimoto *et al*[4] and Soller *et al*[14] also did not observe any *ETV1* chimeric fusions in their small cohorts, supporting our observation that *ETV1* rearrangement is rare. The frequency of *ETV4* gene fusion as reported previously is also rare.[2]



As *TMPRSS2* and *ERG* are located approximately 3 Mb apart in the human genome on chromosome 21, the expression of *TMPRSS2:ERG* fusion transcripts are compatible with either a translocation between chromosome 21s or intrachromosomal deletion. By using break apart assays for both *TMPRSS2* and *ERG*, a deletion spanning from near the 3′ end of *TMPRSS2* to near the 5′ end of *ERG* was identified in 41% (15/37) and 39% (14/36) of cases with rearrangements in the respective genes, confirming the observation by Perner *et al* and Yoshimoto *et al*[4] that intronic loss of genomic DNA between *ERG* and *TMPRSS2* on chromosome 21q22.2–3 is a common mechanism of gene fusion.[3,4] Importantly, 80% (12/15) of cases rearranged for both *TMPRSS2* and *ERG* demonstrated concordant deletion of the 3′ end of *TMPRSS2* and 5′ end of *ERG* (Figures 1A2, 1A3 and 2c).

Interestingly, in 11% (6/57) of cases *TMPRSS2* was rearranged without rearrangement in *ERG*, *ETV1* or *ETV4* (Figure 2b). In these cases, we hypothesized that these cases may harbor rearrangements involving other *ETS* genes family members, which comprise approximately 30 genes. In addition, 1/65 (2%) and 1/58 (2%) cases were rearranged for *ERG* and *ETV 4,* respectively, without rearrangement with *TMPRSS2*, suggesting that other 5′ androgen-regulated partners may be involved with *ETS* partners (Figure 2b). Future work will therefore focus on identifying novel rearrangements that may have biologic or prognostic significance.

The current study identified no statistically significant associations with either *TMPRSS2* or *ERG* rearrangement (with or without deletions) and a higher risk of PSA biochemical failure. However, we did observe a statistically significant association for *TMPRSS2* gene rearrangement and the presence of advanced pathologic tumor stage ($P = 0.04$) (Table 2). Recently, Perner *et al*[3] reported a significant association between tumors with *TMPRSS2:ERG* rearrangements through deletions and higher tumor stage, and presence of pelvic lymph nodes when compared with cancers without *TMPRSS2:ERG* fusions. Petrovics *et al*[15] described *ERG* over expression in a subset of 95 prostate cancer patients and noted that high levels were associated with a variety of different positive prognostic variables such as longer PSA recurrence-free survival, early and intermediate stages, lower pathological T stage,

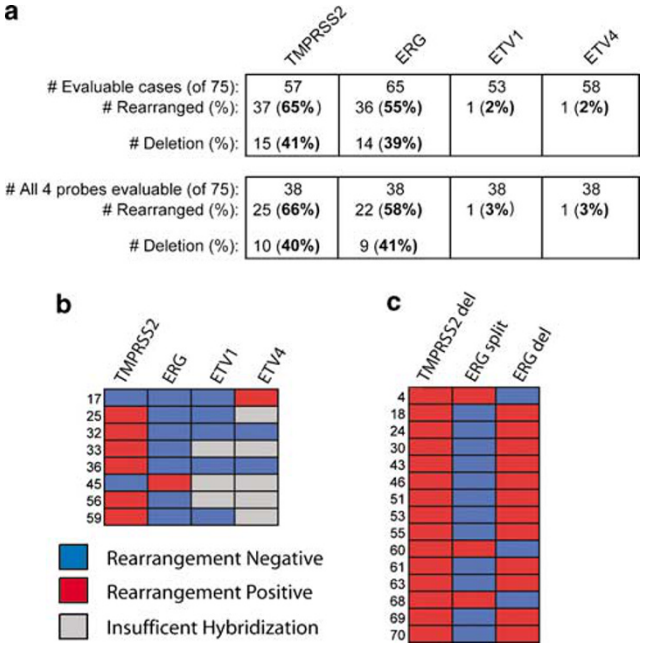


Figure 2 *TMPRSS2*, *ERG*, *ETV1*, and *ETV4* rearrangements as detected by FISH. (**a**) Table of results for rearrangements in *TMPRSS2*, *ERG*, *ETV1* and *ETV4* as detected by the assays shown in Figure 1. Seventy-five of 96 cases were evaluable for at least one assay, and the number of evaluable cases for each assay is indicated. The percentage (of evaluable cases for that assay) and number of cases with rearrangements for each assay is listed. For *TMPRSS2* and *ERG*, the percentage (of rearrangement positive cases) and number of cases with assays consistent with intrachromosomal deletion between *TMPRSS2* and *ERG* are given. The bottom panel contains results when the analysis was limited to the 38 cases where all four probes were evaluable. The number and percentage of these 38 cases with rearrangements for each assay is given, as well as the number and percentage of *TMPRSS2* and *ERG* rearrangement positive cases with intrachromosomal deletion. (**b**) Heat map representation of cases with discordant *TMPRSS2* and *ETS* rearrangement status. (**c**) Heat map representation of cases positive for *TMPRSS2* rearrangement through chromosomal deletion (red) showing concomitant status of *ERG* rearrangement (split or deletion).

Table 1 Clinical and pathological demographics of 96 men with clinically localized prostate cancer treated by radical prostatectomy

	Count	*Column (%)*
Age at diagnosis		
≤60	43	45
>60	53	55
Gleason sum		
<7	35	37
=7	55	57
>7	6	6
Tumor size		
<1 cm	21	22
≥1 cm	75	78
Pathology stage		
T2	73	76
T3a	18	19
T3b	5	5
Surgical margin		
Negative	60	62.5
Positive	36	37.5
Preoperative PSA (ng/ml)		
≤4	19	20
4–7	31	32
>7	46	48
PSA recurrence		
No	62	65
Yes	34	35

PSA, prostate-specific antigen.

Table 2 Clinicopathological associations of prostate cancer cases associated with or without *TMPRSS2* and *ERG* gene rearrangement

Variable	*TMPRSS2− (N = 20)*	*TMPRSS2+ (N = 37)*	P-*value*	*ERG− (N = 29)*	*ERG+ (N = 36)*	P-*value*
Age at diagnosis						
≤60	9 (45%)	15 (41%)	0.75	12 (41%)	17 (47%)	0.64
>60	11 (55%)	22 (59%)		17 (59%)	19 (53%)	
Gleason sum						
<7	9 (45%)	12 (32%)	0.35	12 (41%)	11 (31%)	0.36
≥7	11 (55%)	25 (68%)		17 (59%)	25 (69%)	
Pathology stage						
≤T2b	18 (90%)	24 (65%)	0.04	23 (79%)	22 (61%)	0.11
≥T3a	2 (10%)	13 (35%)		6 (21%)	14 (39%)	
Surgical margin						
Negative	14 (70%)	21 (57%)	0.33	18 (62%)	21 (58%)	0.76
Positive	6 (30%)	16 (43%)		11 (38%)	15 (42%)	
Preoperative PSA (ng/ml)						
≤4	4 (20%)	6 (16%)	0.60	3 (10%)	9 (25%)	0.04
4–7	8 (40%)	11 (30%)		14 (48%)	7 (19%)	
>7	8 (40%)	20 (54%)		12 (41%)	20 (56%)	
EPE						
Negative	18 (90%)	24 (65%)	0.04	23 (79%)	22 (61%)	0.11
Positive	2 (10%)	13 (35%)		6 (21%)	14 (39%)	
Race						
Black	3 (15%)	2 (5%)	0.39	4 (14%)	3 (8%)	0.78
White	16 (80%)	31 (84%)		22 (76%)	29 (81%)	
Unknown	1 (5%)	4 (11%)		3 (10%)	4 (11%)	
PSA recurrence						
No	11 (55%)	22 (59%)	0.75	17 (59%)	23 (64%)	0.66
Yes	9 (45%)	15 (41%)		12 (41%)	13 (36%)	

PSA, prostate-specific antigen.

and negative surgical margins. Wang *et al*[16] suggested that the clinical significance of gene fusions might be related to the splice variants of expressed *TMPRSS/ERG* transcripts, rather than presence of rearrangements alone. They observed a total of eight different isoforms of *TMPRSS2:ERG* fusion transcripts; the expression of certain isoforms, notably type VI, in which the native ATG in exon 2 of the *TMPRSS2* gene is in frame with exon 4 of *ERG* gene and to lesser extent isoforms types I and II were associated with clinical and pathologic variables of aggressive disease. Cancers not expressing these isoforms, but expressing higher levels of fusion mRNAs were also associated with PSA recurrence. Therefore, detailed characterization of these molecular subtypes may further define the biologic significance of recurrent gene fusions in prostate cancer.

One limitation of our study is that majority of prostate cancers in our cohort are characterized by low-stage (pT2 = 76%) cancers with limited representation of high-stage (pT3a = 19% and pT3b = 5%) and high-grade (Gleason score >7 = 6) tumors (Table 1). In addition, studies using PSA biochemical failure as the surrogate end point for the clinical outcomes may not be an adequate measure, in particular for death.[17,18,19] In a recent population-based study of Swedish men with localized prostate cancers followed by expectant (watchful waiting) therapy, Demichelis *et al*[10] observed a statistically significant association between *TMPRSS2:ERG* gene fusions and prostate cancer-specific death. Therefore additional independent studies focusing on larger cohorts using PSA recurrence as well as prostate cancer specific death as an end points may further define overall biologic significance of recurrent gene fusions.

In summary, using *TMPRSS2* break apart probe FISH approach, we demonstrate that approximately 70% of clinically localized prostate cancers in a hospital-based cohort of American men demonstrate chromosomal aberrations, with majority rearranged with the *ETS* partner *ERG*. Importantly, we define a systemic approach to determine the frequency and subtype of these gene rearrangements in prostate cancer. As *TMPRSS2* is constant partner in majority of prostate cancers associated with gene rearrangement, its clinical application as a biomarker or diagnostic test is promising.

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