TMPRSS2-ERG Status Is Not Prognostic Following Prostate Cancer Radiotherapy: Implications for Fusion Status and DSB Repair

Alan Dal Pra, Emilie Lalonde, Jenna Sykes, Fiona Warde, Adrian Ishkanian, Alice Meng, Chad Maloff, John Stigley, Anthony M. Joshua, Gyorgy Petrovic, Theodorus van der Kwast, Andrew Evans, Michael Milosevic, Fred Saad, Colin Collins, Jeremy Squire, Wan Lam, Tarek A. Bismar, Paul C. Boutros, Robert G. Bristow

Departments of Radiation Oncology, Medical Biophysics, Medical Oncology, Laboratory Medicine and Pathology and Biostatistics, University of Toronto, Toronto, ON, Canada
Radiation Medicine Program, Princess Margaret Hospital, University Health Network, Toronto, ON, Canada
Informatics and Bio-Computing Platform, Ontario Institute for Cancer Research, Toronto, ON, Canada
Department of Cancer Genetics and Developmental Biology, British Columbia Cancer Research Centre, Vancouver, BC, Canada
Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada
Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of the Health Sciences, Rockville, MD, USA
Division of Urology, University of Montreal Health Center, Montreal, QC, Canada
Laboratory for Advanced Genome Analysis - Vancouver Prostate Centre, Vancouver, BC, Canada
Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada
Department of Pathology and Laboratory Medicine and Oncology, University of Calgary, Calgary, AB, Canada

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Correspondence to:
Robert G Bristow MD, PhD, FRCPC
Radiation Medicine Program
Princess Margaret Hospital
610 University Avenue
Toronto, ON
M5G 2M9
Canada
Phone: 416-946-2936
Fax: 416-946-4586
Email: rob.bristow@rmpuhn.on.ca

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ABSTRACT

BACKGROUND: Pre-clinical data suggest that TMPRSS2-ERG gene fusions, present in about 50% of prostate cancers (PCa), may be a surrogate for DNA repair status and therefore a biomarker for DNA damaging agents. To test this hypothesis, we examined whether TMPRSS2-ERG status was associated with biochemical failure after clinical induction of DNA damage following image-guided radiotherapy (IGRT). METHODS: Pre-treatment biopsies from two cohorts of intermediate-risk PCa patients (T1/T2, GS < 8, PSA < 20ng/mL) (> 7 years follow-up) were analyzed: (1) 126 patients (CGH-cohort) with DNA samples assayed by array Comparative Genomic Hybridization (aCGH) for the TMPRSS2-ERG fusion; and (2), 118 patients (IHC-cohort) whose biopsy samples were scored within a defined tissue microarray (TMA) immunostained for ERG overexpression (known surrogate for TMPRSS2-ERG fusion). Patients were treated with IGRT with a median dose of 76 Gy. The potential role of TMPRSS2-ERG status as a prognostic factor for biochemical-free relapse rate (bRFR; nadir + 2 ng/mL) was evaluated in the context of clinical prognostic factors in multivariate analyses using a Cox proportional hazards model. RESULTS: TMPRSS2-ERG fusion by aCGH was identified in 27 (21%) of the cases in the CGH-cohort and ERG overexpression was found in 59 (50%) patients in the IHC-cohort. In both cohorts, TMPRSS2-ERG status was not associated with bRFR on univariate or multivariate analysis. CONCLUSIONS: In two similarly-treated IGRT cohorts, TMPRSS2-ERG status was not prognostic for bRFR, in disagreement with the hypothesis that these PCa have DNA repair defects that render them clinically more radiosensitive. TMPRSS2-ERG is therefore unlikely to be a predictive factor for IGRT response.
TRANSLATIONAL RELEVANCE

Improved patient stratification using novel genetic prognosticators or response predictors could help individualize prostate cancer therapies. Pre-clinical studies have shown that TMPRSS2-ERG gene fusion, leading to ERG overexpression, may be a biomarker of DNA double strand break repair (DSB) capacity with potential implications for sensitivity to radiotherapy or DNA damaging modifying agents (e.g. PARP inhibitors).

Using two different technologies (aCGH and IHC), we did not observe that TMPRSS2-ERG status (as assayed in pre-treatment biopsies of intermediate-risk prostate cancer patients) is prognostic for biochemical outcome after image-guided radiotherapy. These clinical results suggest that the presence of a TMPRSS2-ERG fusion is not, de facto, associated with a clinical DSB repair defect that leads to prostate tumor cell radiosensitivity.
INTRODUCTION

Chromosomal rearrangements have a critical role in oncogenic events in prostate cancer (PCa). Tomlins et al. reported a recurrent gene rearrangement involving the 5′ untranslated region of the androgen-regulated TMPRSS2 (transmembrane protease serine 2) gene with ETS (erythroblast transformation specific) gene family members, including ERG (v-ets erythroblastosis virus E26 oncogene homolog [avian], chromosome 21q22.3) or ETV1 (ets variant 1, chromosome 7p21.3)(1). ETS family members are involved in multiple signaling pathways associated with cancer formation and progression (2-4). About 50% of clinically localized PCa cancers harbor TMPRSS2-ERG gene fusions, leading to ERG overexpression(5). Newer immunohistochemical (IHC) approaches using ERG-specific antibodies have shown that ERG protein overexpression in situ is a sensitive and specific surrogate for the presence of TMPRSS2-ERG gene fusion detected by FISH or qRT-PCR (1, 6-8). If the presence of a fusion, or ERG overexpression, is associated with differential prognosis or treatment response this would have major implications for its clinical use in a cancer that is diagnosed in more than 250,000 men in North America each year(9).

Intermediate-risk PCa is defined by National Comprehensive Cancer Network (NCCN) as T1/T2-N0-M0 with a Gleason score 7 and PSA < 20 ng/mL or Gleason score < 7 and PSA 10-20 ng/mL.(10) Clinical outcomes are highly heterogeneous within this risk category, with up to 30-40% of patients failing therapy independent of treatment modality (11, 12). Therefore, identification of additional prognostic factors that could stratify these patients into more precise prognostic or predictive sub-groups based on individual tumor genetics factors would be extremely valuable.
Studies addressing the relationship between *TMPRSS2-ERG* gene fusions and PCa aggression or clinical outcome have provided conflicting results (13-18). However, in the largest cohort tested to date ERG overexpression (determined by IHC) was not prognostic for biochemical recurrence following radical prostatectomy (19). This lack of prognostic significance in surgery patients was confirmed by a recent meta-analysis using biochemical recurrence and disease-specific mortality as endpoints (20). However, the role of *TMPRSS2-ERG* as a response modifier in patients receiving modern era radiotherapy has not yet been evaluated.

Precision radiotherapy delivered with image-guidance (IGRT) is an important modality for PCa treatment. Recent pre-clinical data suggest that that *TMPRSS2-ERG* status may relate to DNA repair and radiotherapy-induced DNA damage. Using fluorescence in situ hybridization (FISH), androgen signaling was found to induce proximity of the *TMPRSS2* and *ERG* genomic loci (both located on chromosome 21q22.2), particularly following induction of DNA double-strand breaks (DSBs) by irradiation or inhibition of topoisomerase II beta (TOP2B) (21, 22). Other data support fusion status associated with altered sensitivity to DNA damaging agents(23). Stable overexpression of *TMPRSS2-ERG* fusion product in PCa cells can alter radiosensitivity, and *TMPRSS2-ERG* fusion status can render tumor cells sensitive to poly (ADP-ribose) polymerase 1 (PARP1) inhibition *in vitro* and *in vivo*(24). In the latter study, the *TMPRSS2-ERG* fusion products interacted in a DNA-independent manner with PARP1 and the catalytic subunit of DNA protein kinase, a DSB repair protein. The authors concluded that overexpression of the *TMPRSS2-ERG* fusion induces DNA damage, which is potentiated by PARP inhibition (PARPi) and leads to cell death. This was
similar to the cell death observed in PARPi-treated cells defective in the homologous recombination (HR) pathway of DSB repair.

Taken together, these pre-clinical data suggest that the *TMPRSS2-ERG* status of primary PCa may reflect relative *a priori* DNA repair capacity, and thus could alter the therapeutic response to DNA damaging agents, including precision radiotherapy. If true, PCa gene fusion status could be predictive for treatment outcome. We therefore tested the ability of *TMPRSS2-ERG* status to predict outcome in intermediate-risk PCa patients following clinically-induced DSBs using image-guided radiotherapy (IGRT).

MATERIALS AND METHODS

Patient Cohorts and Treatment Delivery

We investigated *TMPRSS2-ERG* status in pre-treatment biopsies of intermediate-risk PCa patients using two different methods in two different cohorts: 1) *TMPRSS2-ERG* gene fusion assessed at the DNA level using array comparative genomic hybridization (aCGH); or 2), ERG protein overexpression assayed by IHC. Both cohorts included patients who completed curative radical radiotherapy for histologically-confirmed adenocarcinoma of the prostate as part of prospective clinical studies approved by the University Health Network Research Ethics Board and registered (NCT00160979; ISRCTN64733264) in accordance with the criteria outlined by the International Committee of Medical Journal Editors. This work followed the REMARK recommendations for tumor marker prognostic studies (25) (Supplementary Table 1). The aCGH-cohort consisted of 126 evaluable patients; further details on the assay technique
and background tumor genetics for this cohort have been described previously (26). Clinical characteristics for both aCGH- and IHC-cohorts are presented in Table 1. To create the IHC-cohort, formalin-fixed paraffin-embedded (FFPE) pre-treatment biopsies from 173 patients were used to construct a biopsy tissue microarray (TMA). Post-array, the cohort was reduced to 118 evaluable patients after a quality assurance protocol, which removed patients if malignant cores could not be scored within the histologic section; if the NCCN criteria of intermediate risk disease was not met (27); or if they lacked follow-up data (see Figure 1B).

For both cohorts, patients underwent trans-rectal ultrasound (TRUS)-guided insertion of three intra-prostatic gold fiducial markers for radiotherapy planning and IGRT. Research biopsies (two for formalin-fixation and one fresh-frozen in liquid N₂) were taken during fiducial marker insertion. Staging CT and bone scans were not routinely performed. The clinical target volume (CTV) encompassed the prostate gland alone. The planning target volume (PTV) was defined by a 10 mm margin around the CTV, except posteriorly where the margin was 7 mm. All patients were treated with 6-field conformal or intensity-modulated radiotherapy (IMRT) with image-guidance. The radiotherapy dose was variable within the two cohorts, so doses were converted to biologically effective doses (BED) with an assumed alpha-beta of 1.5 (28). Dose details are presented in Table 1 for both cohorts. Neoadjuvant and concurrent hormonal therapy (ADT) was used in 33 patients (26%) in the aCGH-cohort and in 35 patients (29%) in the IHC-cohort. This ADT consisted of bicalutamide 150mg daily for 3 months of neoadjuvant treatment followed by a further 2 months as concurrent treatment with radiotherapy (ISRCTN64733264) (29). Patients were followed at 6 monthly intervals.
after completing treatment with clinical examination and PSA testing. Additional tests and the management of patients with recurrent disease were at the discretion of the treating physician. The median follow-up of surviving patients was 7.8 and 7.2 years following the start of radiotherapy for the aCGH- and IHC-cohorts, respectively.

*aCGH analysis*

The biopsy preparation, DNA extraction, aCGH procedure, and copy number detection were previously described (26, 30). For each patient, the presence of a *TMPRSS2-ERG* gene fusion was defined as an observation of a 21q22.2-3 genomic deletion. More specifically, a deletion must overlap with the region contained by the 5’ and 3’ ends of *ERG* and *TMPRSS2*, respectively (Supplementary Figure 1).

*Tissue microarray (TMA) and Immunohistochemistry*

The biopsy TMA was constructed from pre-treatment prostate biopsies using a “checkerboard” technique as previously described (32). Benign and malignant prostate tissues within each core were denoted for dissection based on hematoxylin and eosin (H&E)-stained sections by an experienced genitourinary pathologist (TVdK). Based on pathologic markings, 4 mm long “checkers” were cut along the length of the biopsies and flipped 90 degrees and placed within a TMA template (see Supplementary Figure 2). Although 173 patients had diagnostic biopsy blocks available, a pathologic re-assessment was completed in which each checker was confirmed between contiguous slices for the presence or absence of malignancy. After this quality assurance step, and after removing patients lacking follow-up data or who did not present with intermediate-risk disease, a
total of 118 patients remained for comparison to clinical parameters and outcome (Figure 1A). An assessment of the intra-patient heterogeneity of number of checkers is shown in Figure 2 as evaluated using the Kappa and Fleiss Kappa approaches (33, 34). This analysis showed that for patients with more than one checker there was significant agreement between ERG staining results.

Immunostaining of the tissue microarrays for ERG was performed as follows: deparaffinized 4 μm sections were dehydrated, blocked in 0.6% hydrogen peroxide in methanol for 20 minutes and processed for antigen retrieval in EDTA (pH 9.0) for 30 minutes in a microwave, followed by 30 minutes of cooling in EDTA buffer. Sections were then blocked in 1% horse serum followed by an overnight incubation with the ERG–MAb mouse monoclonal antibody (Biocare Medical clone 9Fy, Concord, CA), diluted 1:300 at room temperature. The immunostaining was developed using the Polymer-HRP IHC kit (Biogenex, Fremont, CA) according to manufacturer's instructions. Next, sections were counterstained in hematoxylin for 1 minute, dehydrated, cleared and mounted. Immunostained TMA checkers were evaluated for ERG staining based on the presence or absence of positive nuclear immunoreactivity in prostatic adenocarcinoma cells relative to endothelial cells nuclei (which served as a positive control) (see Figure 1B). Checkers with faint or negative endothelial cell staining were excluded from analysis. ERG expression was then dichotomized for positive and negative expression. We considered a case positive for ERG expression if any of the replicate checkers from that case showed any positive ERG staining.
Statistical analysis

The primary outcome was biochemical relapse-free rate (bRFR) defined according to Phoenix criteria (PSA nadir + 2 ng/mL) (35) or institution of salvage ADT (patients treated with ADT by their attending physician due to serial and rising PSA values, post-IGRT). Time to biochemical failure was measured from the start of treatment until the date of biochemical failure or date of last PSA measurement. Five-year biochemical relapse-free rates were calculated using the Kaplan-Meier method. The associations between either TMPRSS2-ERG fusion or ERG overexpression and clinical factors were examined, using the Fisher’s exact test for Gleason score and T category, and the Mann-Whitney test for pre-treatment PSA. The log-rank test was used to compare relapse rates between patients with and without TMPRSS2-ERG fusion or ERG overexpression. The effects of TMPRSS2-ERG fusion and ERG overexpression on bRFR were also tested adjusting for pre-treatment PSA, T category, and Gleason score using Cox proportional hazards regression models. The proportional hazards assumption was checked using Schoenfeld residuals and found to be satisfied for all variables, with the exception of ADT in the aCGH cohort. A time-varying coefficient was added to the Cox model to account for this model violation. All statistical analyses were done using the R statistical environment (v2.12.1). Hazard ratios, 95% confidence intervals and p-values using the Wald test were generated using the survival package version (v2.36-5). A two-sided p-value of less than 0.05 was used to assess statistical significance.
RESULTS

We designed this study to test whether IGRT patients had a differential prognosis based on fusion status. If true, fusion status would become a novel predictive factor for outcome in patients receiving radiotherapy (but not surgery). The clinical characteristics of both aCGH and IHC-cohorts are presented in Table 1. These cohorts were comprised by intermediate-risk patients mostly with T2 disease, Gleason score 7 and PSA less than 10 ng/mL. The mean radiation dose was 76Gy. In the aCGH-cohort, 27 of 126 biopsies (21%) were found to be TMPRSS2-ERG fusion-positive. In the IHC-cohort, a positive ERG immunohistochemical staining was observed in 59 (50%) of the cases. We next tested whether fusion status was associated with more aggressive clinical states in our IGRT cohort. ERG overexpression was associated with T-category (T2 vs. T1; P = 0.02); but not with Gleason score (7 vs. 6; P = 1.00) or pre-treatment PSA (continuous, P = 0.28). TMPRSS2-ERG fusion (aCGH-cohort) was not correlated to any of these clinical variables (Supplementary Tables 2A-F).

We then tested fusion status as a prognostic factor for biochemical failure following IGRT. At a median follow up of 7.8 years (range 0.8–12.2), 55 patients (44%) in the aCGH-cohort experienced biochemical relapse (see Table 1). Of these, 20 had biopsy-proven local failure in which 5 were fusion positive and 15 fusion negative (a similar proportion to the entire cohort and arguing against fusion status associated with increased radioresponse). For the IHC-cohort, at a median follow-up of 7.2 years (range: 0.33 – 12.2), 31 (26%) patients presented biochemical failure. Of the 31 patients with biochemical failure in this cohort, 8 had biopsy-proven local failure (3 were ERG positive
and 5 ERG negative); again showing no trend for ERG overexpression to be associated with increased radioresponse.

The prognostic significance of pre-treatment PSA, T-category and Gleason score for bRFR for both cohorts is shown in Table 2 and Supplementary Figure 3A-F. Only pre-treatment PSA in the aCGH-cohort was prognostic for bRFR. We then added data pertaining to fusion status into the model. *TMPRSS2-ERG* status, whether assayed by aCGH or IHC, was not prognostic for bRFR following radiotherapy in either univariate or multivariate analyses (see Figure 3A-B, respectively). The univariate hazard ratios (HR) associated with *TMPRSS2-ERG* in the aCGH- and IHC-cohorts were 0.78 (95% CI: 0.41-1.49; p = 0.46) and 0.99 (95% CI: 0.48-2.02; p = 0.97), respectively.

In concert with other publications showing the potential predictive value of *TMPRSS2-ERG* status on androgen deprivation therapy response (18, 37, 38), ERG overexpression was reported to be a factor in the relative response to salvage ADT following surgery (19). However, in our subgroup of patients treated with ADT, neither *TMPRSS2-ERG* fusion nor ERG overexpression predicted outcome. Additionally, the analysis of those patients treated without ADT also showed no predictive value of TMRSS2-ERG status. (See hazard ratio values associated with Kaplan-Meir plots in Supplementary Figures 4 and 5).

Given our goal to analyze IGRT patient outcome on the basis of aCGH or ERG overexpression as a prognostic versus predictive factor, we additionally determined whether fusion status was prognostic in a radical prostatectomy cohort in a similar low-to intermediate-risk cohort using a published dataset (36). Details for this surgical cohort were previously described (26). In this cohort, neither TMRSS2-ERG fusion (by aCGH)
nor ERG overexpression (based on mRNA abundance) were prognostic in 131 men with a median follow-up of 4.6 years (see Supplementary Figure 6 and 7). Therefore, our studies suggest that TMPRSS2-ERG status is not prognostic in intermediate-risk patients treated with IGRT or radical prostatectomy.

DISCUSSION

To our knowledge, this is the first study to address the role of TMPRSS2-ERG status in pre-treatment biopsies of PCa patients treated with radical radiotherapy, one of the main treatment options for this disease. This is a pre-requisite to using this information to personalize treatment at the time of diagnosis. Our clinical data shows that TMPRSS2-ERG status, assayed using ERG overexpression or by aCGH, is not prognostic factor for biochemical recurrence after IGRT. This was also true for a small sub-group of patients treated with neoadjuvant and concurrent high-dose (150 mg per day) bicalutamide. Given recent data in surgical cohorts, this suggests that TMPRSS2-ERG status is not a determinant of recurrence following precision local therapies.

There are two main pathways of DSB repair: (1) non-homologous end-joining (NHEJ) in which a defect in this pathway leads to profound radiosensitivity and (2) homologous recombination (HR) in which less profound, but still appreciable, radiosensitivity is observed. (39) As such, if the fusion was associated with a defect in NHEJ or HR, we would have observed a profound and durable PSA response in fusion-positive PCa relative to fusion-negative PCa. Given that TMPRSS2-ERG status is not predictive for radiotherapy response, our clinical study does not support the pre-clinical hypothesis that fusion-positive, localized PCa is functionally deficient in DSB repair to
the extent that is clinically-relevant for an IGRT treatment effect (21, 23, 24). Additionally, although limited data was available for the CGH- and IHC-cohorts, there was no evidence that post-radiation, biopsy-positivity was less in fusion-positive patients compared to fusion-negative patients. Finally, unlike the prognostic role of MYC amplification and/or loss of PTEN or NKX3.1 alleles (26, 40), fusion positivity does not lead to rapid early failure post-IGRT (suggestive of an association with occult metastases and relatively aggressive disease at the time of local treatment).

Our study has a number of limitations. Given the multi-focality and molecular heterogeneity of PCa, one possible weakness of our analysis is that aCGH data was based on only one biopsy to an index lesion (26) and 78% of the cases from IHC cohort had only one checker assayed. Although there is evidence showing that the dominant lesion is the most common location for recurrence post-treatment (41) and one core per tumor can be sufficient (42, 43), we cannot rule out that we have under-called fusion status in this aCGH-cohort (44). However, our additional and complementary analysis of 118 intermediate-risk patients in the IHC-cohort showed ERG overexpression in 50% of those tumors. This is in the range of previous series assessing ERG expression by IHC, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) or Fluorescence In Situ Hybridization (FISH) (8, 45, 46). Furthermore, we have shown that when more than one checker per patient was available there was little intra-patient heterogeneity for ERG status (Figure 2). Finally, given the confidence intervals for HR as shown in Table 2A (aCGH cohort with CI: 0.40-1.55) and 2B (IHC cohort with CI: 0.42-1.88), this study can only rule out affect sizes on HR less than 0.40-0.42, or greater than 1.55-1.88, in two cohorts. However, in the latter case, values greater than 1.0 would be associated with
increasing risk of failure following IGRT (i.e. radioresistant phenotype) which would still argue against the hypothesis that fusion status is a marker of defective DSB repair associated with tumor cell radiosensitivity.

In the future, it would be advantageous to collect post-IGRT biopsies for all patients in order to better define the role of fusion status in terms of local control versus systemic relapse. Karnes et al. have proposed that ERG positive patients present a better response to androgen deprivation (38) and recently, *TMPRSS2-ERG* status has been shown to be a predictive biomarker for androgen therapy in the form of abiraterone.(47) As such, our results could differ in patients receiving combined-modality therapy as the primary treatment (e.g. high-risk or locally-advanced PCa) in which fusion status could be studied in the context of the need for salvage ADT (including enzalutamide or abiraterone) or systemic chemotherapy. These concepts could be investigated in tissues prospectively collected in randomized clinical trials.

Recent evidence suggests that gene rearrangements involving *TMPRSS2* and the ETS transcription factor *ETV1* drive a distinct transcriptional program compared to *TMPRSS2-ERG*. In the context of *PTEN* deletion, these tumors seem to have more aggressive disease and poorer outcome.(48) Furthermore, a quantitative assessment of ETV1 overexpression (48) and ERG overexpression (18) (rather than positive or negative) has been reported to be prognostic across risk groups. Future studies using pretreatment biopsies could test these endpoints in TMAs where PCa cellularity is increased to the extent that quantitative immunohistochemistry is possible (e.g. possibly high-risk prostate cancers). In our intermediate risk series, selected samples had fewer than 50 cells and therefore quantitative scoring of expression was not deemed feasible.
Molecular prognostic and prediction is an important requirement in novel approaches to personalized cancer medicine. Only large prospective IGRT and dose-escalated cohorts, which also document the presence or absence of *TMPRSS2-ERG* gene fusion, will define its complete role in PCa treatment. Additional clinical studies are required to understand the potential complex relationship between *TMPRSS2-ERG* gene fusion, functional DNA repair, AR expression (49) and clinical outcome following treatment with agents that modify the DNA damage response, including PARP inhibitors.
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REFERENCES


**TABLES**

Table 1: Clinical characteristics of aCGH and IHC treatment cohorts

<table>
<thead>
<tr>
<th></th>
<th>IHC Cohort (n = 118)</th>
<th>aCGH Cohort (n = 126)</th>
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<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td><strong>T-score</strong></td>
<td></td>
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<tr>
<td>T1</td>
<td>43 (36%)</td>
<td>45 (36%)</td>
</tr>
<tr>
<td>T2</td>
<td>75 (63%)</td>
<td>81 (64%)</td>
</tr>
<tr>
<td><strong>Gleason</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>29 (24%)</td>
<td>31 (25%)</td>
</tr>
<tr>
<td>7</td>
<td>89 (74%)</td>
<td>95 (75%)</td>
</tr>
<tr>
<td><strong>Pre-treatment PSA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10</td>
<td>79 (66%)</td>
<td>88 (70%)</td>
</tr>
<tr>
<td>&gt;10</td>
<td>39 (33%)</td>
<td>38 (30%)</td>
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<tr>
<td>Median (range)</td>
<td>7.7 (1.3-19.6)</td>
<td>7.8 (0.9-19)</td>
</tr>
<tr>
<td><strong>ADT</strong></td>
<td>35 (29%)</td>
<td>33 (26%)</td>
</tr>
<tr>
<td><strong>RT dose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 Gy/20fr</td>
<td>7 (6%)</td>
<td>12 (10%)</td>
</tr>
<tr>
<td>66 Gy/22fr</td>
<td>4 (3%)</td>
<td>3 (2%)</td>
</tr>
<tr>
<td>75.6 Gy/42fr</td>
<td>27 (23%)</td>
<td>33 (26%)</td>
</tr>
<tr>
<td>78 Gy/39fr</td>
<td>4 (3%)</td>
<td>3 (2%)</td>
</tr>
<tr>
<td>79.8 Gy/42fr</td>
<td>76 (63%)</td>
<td>75 (60%)</td>
</tr>
<tr>
<td>Mean equivalent dose&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.4 Gy</td>
<td>76 Gy</td>
</tr>
<tr>
<td><strong>Biochemical failures&lt;sup&gt;b&lt;/sup&gt;</strong></td>
<td>31 (26%)</td>
<td>55 (44%)</td>
</tr>
<tr>
<td><strong>Deaths</strong></td>
<td>12 (10%)</td>
<td>7 (5%)</td>
</tr>
<tr>
<td><strong>Median FU (years)</strong></td>
<td>7.2</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>(0.33-12.2)</td>
<td>(0.8-12.2)</td>
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<sup>a</sup>Mean equivalent dose was calculated using Biologically Effective Dose (BED) formula at 2Gy-daily fractions with an α/β ratio of 1.5 for tumor response.

<sup>b</sup>As defined by Phoenix criteria (PSA nadir + 2ng/ml); except an additional 5 patients in the aCGH-cohort that were pre-emptively treated with salvage ADT due to rising PSA post-treatment;

IHC=immunohistochemistry; aCGH=array Comparative Genomic Hybridization; ADT=androgen deprivation therapy; RT=radiotherapy; FU=follow-up;
Table 2: Multivariate analysis of clinical prognostic factors for bRFR in the aCGH- and IHC-cohorts

A) Clinical model, aCGH-cohort

<table>
<thead>
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<th></th>
<th>HR</th>
<th>95% Conf Int</th>
<th>p-value</th>
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<tr>
<td>T category: 2 vs. 1</td>
<td>1.02</td>
<td>0.56</td>
<td>1.85</td>
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<tr>
<td>PSA (continuous)</td>
<td>1.13</td>
<td>1.05</td>
<td>1.21</td>
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<tr>
<td>Gleason 7 vs. 6</td>
<td>0.93</td>
<td>0.49</td>
<td>1.77</td>
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<tr>
<td>ADT</td>
<td>0.16</td>
<td>0.03</td>
<td>0.87</td>
</tr>
<tr>
<td>ADT with time</td>
<td>1.03</td>
<td>1.01</td>
<td>1.05</td>
</tr>
<tr>
<td>Fusion positive</td>
<td>0.79</td>
<td>0.40</td>
<td>1.55</td>
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B) Clinical model, IHC-cohort

<table>
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<th>HR</th>
<th>95% Conf Int</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>T category: 2 vs. 1</td>
<td>2.16</td>
<td>0.90</td>
<td>5.23</td>
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<tr>
<td>PSA (continuous)</td>
<td>1.06</td>
<td>0.97</td>
<td>1.15</td>
</tr>
<tr>
<td>Gleason 7 vs. 6</td>
<td>1.32</td>
<td>0.52</td>
<td>3.34</td>
</tr>
<tr>
<td>ADT</td>
<td>0.92</td>
<td>0.42</td>
<td>2.05</td>
</tr>
<tr>
<td>ERG positive</td>
<td>0.89</td>
<td>0.42</td>
<td>1.88</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. A) Study flowchart. After exclusion of 55 patients, a total of 118 patients were available for analysis. B) Representative images of ERG immunohistochemistry in a ERG negative checker in a Gleason score 7 prostate adenocarcinoma (left) showing positive endothelial cells as control (arrow); and a ERG positive checker in a Gleason score 7 (right).

Figure 2. Frequency-histogram showing the distribution of the 118 patients according to ERG staining (positive vs. negative), presence of malignant tissue in the checker (no benign tissue was ERG positive) and number of checkers per patient (ranging from 1 to 3). Table below depicts the analysis of intra-patient heterogeneity of checkers. Eighty percent (95/118) of the patients had information on a single biopsy checker. There were 20 patients that had ERG scored on 2 checkers, and 3 patients that had ERG scored on 3 checkers. Cohen’s Kappa (33) was used to assess patients with 2 checkers and Fleiss’ Kappa (34), 3 checkers. Patients with 2 checkers were in perfect agreement (Cohen’s $\kappa = 1$, $P < 0.0001$), while for the 3 patients with 3 checkers, the checkers were in moderate agreement ($\kappa = 0.55$, $P = 0.099$).

Figure 3. Univariate Kaplan-Meier plots of biochemical relapse-free rates (bRFR) versus TMPRSS2-ERG status in the aCGH-cohort (A) and IHC-cohort (B).
Figure 1.

173 patients

55 patients excluded from analysis due to:
- Benign tissue (32);
- Checkers fall off (12);
- No follow-up data (1);
- High-risk category (10);

118 patients

59 patients

ERG +

59 patients

ERG -

A

B
Figure 2.

ERG Status

<table>
<thead>
<tr>
<th>Number of Checkers</th>
<th>ERG Status (positive vs. negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
</tr>
</tbody>
</table>

- Two checkers, n=20, Cohen’s $\kappa = 1$, $p<0.0001$
- Three checkers, n=3, Fleiss’ $\kappa = 0.55$, $p=0.099$
Figure 3A.

Fusion (+) vs. Fusion (-)
HR=0.78, 95% CI: 0.41-1.49
Log-rank p-value=0.46

- Fusion (-), n=99, 5y RFR=73%
- Fusion (+), n=27, 5y RFR=80%

Time to biochemical relapse (months)
Figure 3B.

ERG Positive vs. ERG Negative, HR=0.99, 95% CI: 0.48–2.02
Log-rank p-value=0.97

- ERG Negative, n=59, 5y RFR=83%
- ERG Positive, n=59, 5y RFR=94%
Clinical Cancer Research

TMPRSS2-ERG Status Is Not Prognostic Following Prostate Cancer Radiotherapy: Implications for Fusion Status and DSB Repair

Alan Dal Pra, Emilie Lalonde, Jenna Sykes, et al.

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