Published in final edited form as:

Cancer Res. 2010 December 1; 70(23): 9544–9548. doi:10.1158/0008-5472.CAN-10-1638.

Androgen-induced *TMPRSS2:ERG* fusion in non-malignant prostate epithelial cells

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Abstract

Fusion genes play important roles in tumorigenesis. The identification of the high frequency *TMPRSS2* fusion with *ERG* and other *ETS* family genes in prostate cancer highlights the importance of fusion genes in solid tumor development and progression. However, the mechanisms leading to these fusions are unclear. We investigated whether androgen, through stimulating its receptor, can promote spatial genome reorganization and contribute to the generation of the *TMPRSS2:ERG* fusion. We show that treatment with androgen can induce the *TMPRSS2:ERG* fusion in both malignant and non-malignant prostate epithelial cells. While the fusion could be detected in malignant cells following 24 hours treatment, prolonged exposure to androgen was required to detect the fusion transcript in non-malignant cells. We associated the fusion incidence with genetic factors, including androgen-induced gene proximity, androgen receptor exon1 CAG repeat length and expression of the *PIWIL1* gene. This study demonstrates that fusions can be induced prior to malignant transformation and generation of the fusion is associated with both gene proximity and loss of the ability to prevent double-strand breaks.

Keywords

prostate cancer; fusion gene; gene proximity; AR activity; *PIWIL1* expression

Introduction

Gene fusion events are a hallmark of human haematological and soft tissue malignancies and can be associated with tumor development, progression and therapeutic response(1, 2). Recently, gene fusions involving the *ETS* transcription factor gene family members, including *ERG*, *ETV1*, *ETV4* and *ETV5* have been identified as common events in prostate cancer and highlight the importance of fusion genes in the development and progression of epithelial cancers(2-4). However, the mechanism underlying the development and recurrence of these fusion genes is not yet fully understood.

Androgen, through stimulating its receptor and activating downstream cellular processes, is essential for normal prostate development and prostate cancer cell proliferation(5). Upon androgen binding, androgen receptor (AR), a nuclear transcription factor, is activated and regulates the transcription of a number of genes including *TMPRSS2*(5, 6). The

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TMPRSS2:ERG fusion, detected in approximately 50% of prostate cancers, is the most common fusion gene found in human malignancies(2, 4). This fusion results in over-expression of the *ERG* oncogene from the androgen-regulated *TMPRSS2* promoter. Recently, estrogen has been shown to induce rapid chromosome interactions involving estrogen receptor transcription units(7). Transcriptional activity can lead to the proximity of distal genes(7-9) and gene proximity of fusion partner genes has been associated with fusion events(6, 8-10). As androgen stimulates the expression of *TMPRSS2*, it may induce the proximity and fusion of *TMPRSS2* and its fusion partners. Here we show that AR activity promotes *TMPRSS2:ERG* fusion in non-malignant cells by long-term androgen treatment of immortalized prostate epithelial cells. Furthermore, induced fusion events are associated with both gene proximity and expression of *PIWIL1*, which is thought to have a role in protecting cells from DNA double-strand breaks (DSBs).

Materials and Methods

Cultured cells and clinical samples

Cell lines, including the SV40-immortalized prostate epithelial cell lines PNT1a and PNT2 (11) (obtained from Norman Maitland and Colin Cooper, respectively) and prostate cancer cell lines LNCaP, DU145, PC3, 22RV1 and VCaP (ATCC), were routinely cultured in Dulbecco's Modified Eagle's Media (DMEM) with 10% fetal bovine serum. They were tested and authenticated in August 2010 using ABI AmpF/TSR Identifier kit. Normal prostate epithelial cells (PrEC) (Lonza) were cultured in Clonetics PrEBM medium (Lonza). Primary prostate cancer samples were obtained from The Barts and The London Hospital NHS Trust and Whipps Cross Hospital in London with ethical approval and patients' consent.

Dihydrotestosterone (DHT) treatment

For co-localization analysis following short-term DHT treatment, cells were cultured in RPMI medium with 10% carbon stripped serum (Autogen Bioclear) for 24 hours. Cells were treated for three hours with 100 nM DHT or, cultured under the same conditions, without DHT. For long-term treatment, cells were cultured in DMEM medium with 10% standard fetal bovine serum and additional 300 nM or 3000 nM DHT. Medium was changed twice weekly and fresh DHT added each time.

AR microsatellite analysis

AR CAG repeat length was determined by microsatellite analysis. PCR using primers (AR_FAM_F: 5'-FAM-ACCCAGAGGCCGCGAGCGCAG and AR_R: 5'-TTGCTGTTCCTCATCCAGGA) (Sigma), flanking the AR CAG repeat in exon1 was performed. PCR product was run on 3730xl ABI sequencer and analyzed by Genemapper v 4.0 (Applied Biosystems).

DNA sequencing

PCR product was sequenced using the 3730xl ABI sequencer. Primers pairs *TMPRSS2_F* (5'-GGAGCGCCGCTGGAG) and *ERG_R* (5'-CCATATTCTTTACCGCCAC), and *AR_F* (5'-ACCCAGAGGCCGCGAGCGCAG) and *AR_R* (Sigma) were used to confirm *TMPRSS2:ERG* fusion products and CAG repeat length, respectively.

Gene expression analysis

RNA was extracted with TRIzol (Invitrogen). cDNA was synthesized from RNA using Superscript II (Invitrogen) following the manufacturer's protocols.

Relative mRNA levels were determined using pre-designed Taqman® gene expression assays targeting *PIWIL1* (Hs00380305_m1) and *GAPDH* (Hs99999905_m1) (Applied Biosystems) and quantified by Q-RT-PCR analysis using the ABI Prism 7700 Sequence Detector (Applied Biosystems).

TMPRSS2:ERG fusion transcripts were detected by nested RT-PCR using primers described previously(12). cDNA synthesized from 100 ng of total RNA was used as starting template.

Fluorescence *in situ* hybridization (FISH)

TMPRSS2 and *ERG* co-localization analysis was performed using standard FISH protocol with two bacterial artificial chromosomes (BACs), RP11-35C4 (*TMPRSS2*) and RP11-476D17 (*ERG*). BAC DNA was amplified using GenomiPhi amplification V2 kit (GE Healthcare) and labeled with digoxigenin and biotin, respectively, using the BioPrime labeling kit (Invitrogen). A minimum of 100 nuclei were counted per sample. Induced proximity was quantified and represented as percentage of co-localized signal pairs.

Cell cycle analysis

Cell cycle distribution was established using a FACScalibur flow cytometer (Becton Dickinson). Cells were harvested after 24 hours and stained using propidium iodide (50 µg/ml). The distribution of cell cycle phases was quantified using the WinMDI v2.8 program (<http://facs.scripps.edu/>).

γ-H2AX foci counting

γ-H2AX foci were detected using a monoclonal anti-phospho-Histone H2A.X antibody (clone JBW301, Millipore). Cells exhibiting more than five foci were considered positive. A minimum of 100 cells were counted.

Cell transfection

LNCaP cells were transfected with *PIWIL1* expression plasmid (Origene) using Nucleofector(r) Kit R (Lonza) according to the manufacture's protocol.

Statistical analysis

Differences in the frequency of co-localization signals between groups were compared using a two-tailed Chi-squared test. P values of < 0.05 were considered statistically significant.

Results and Discussion

Androgen can induce *TMPRSS2:ERG* gene fusion in both malignant and non-malignant prostate cells

Prostate cancer develops years after androgen levels peak, indicating that long-term androgen exposure may be required in prostate cancer development. Thus, we treated immortalized non-malignant cell lines, PNT1a and PNT2, continuously with high doses (300 nM and 3000 nM) of the AR ligand DHT. After five months treatment, we detected the fusion transcript in both cell lines treated with 3000 nM DHT and PNT2 cells treated with 300 nM DHT using nested RT-PCR (Fig. 1A). For both PNT1a and PNT2, sequencing of the PCR products revealed the most common fusion isoform; exon1 of *TMPRSS2* fused to exon4 of *ERG*(6, 12) (Fig. 1B). No fusion was detected in cells cultured without DHT. The cell cycle distribution and DNA damage levels, (as determined by γ-H2AX formation) were unaffected by the long-term treatment (Supplementary Figs. S1 and S2). The fusion was induced more frequently in cells treated with higher doses (Fig. 1A), suggesting induction is dosage associated. We treated cells with 100 nM DHT for 24 hours and detected fusion

transcripts in LNCaP but not PrEC, PNT1a and PNT2 cells (Fig. 2A and B). As the long pathogenesis of prostate cancer *in vivo* suggests that physiological DHT doses would be unable to induce *TMPRSS2:ERG* in the short term, the doses used exceeded normal physiological levels, similar to previous studies(6, 13, 14).

During the course of this study, it was reported that DHT treatment could induce fusion events(6, 14). However, as cells were treated for a maximum of 24 hours in the previous studies, DNA damaging agents were used in combination with DHT to induce the fusion events(6, 14). With DHT alone, the fusion could be detected in LNCaP, but not PrEC cells(6). This was confirmed by our short-term treatment of non-malignant cells and may explain why long-term treatment is required to induce the fusion in immortalized prostate epithelial cells. Our finding that *TMPRSS2:ERG* can be induced in pre-malignant prostate epithelial cells is important and suggests that androgen-induced fusions represent an event that can occur prior to prostate cancer development, rather than as a secondary genetic change after the cancer is established. While *TMPRSS2:ERG* has previously been observed in non-malignant prostate cells(12) and cancer precursor lesions(15-18) our results suggest that the fusion may be a consequence of long-term androgen exposure.

***TMPRSS2:ERG* is associated with AR activity**

Our data, indicating that the frequency of androgen-induced *TMPRSS2:ERG* fusion events is dose dependent, suggests that the fusion is more likely induced in individuals with high prostate DHT levels. Alternatively, it is possible that a high frequency of fusion events may occur as a result of increased AR activity. As the length of CAG repeats in exon1 of the *AR* gene varies in individuals and is inversely correlated with AR activity(5), we investigated if this is associated with the occurrence of the *TMPRSS2:ERG* fusion. We determined repeat length in 40 prostate cancer clinical samples, for each of which the *TMPRSS2:ERG* fusion status has been determined by RT-PCR. Overall, repeat length of the fusion-positive cases (mean=20.3) showed a trend to be shorter than fusion-negative cases (mean=21.3) (Supplementary Table S1 and Fig. S3). However, this difference is not statistically significant ($P=0.14$). A large series of samples should be investigated to verify this. We have recently shown that *TMPRSS2:ERG* occurs less frequently in Chinese than Western prostate cancer samples(19). Interestingly, compared to Western males, Chinese men have lower androgen levels and a longer CAG repeat length(20, 21). This difference may account for the low incidence of prostate cancer in China.

DHT stimulation of AR induces *TMPRSS2* and *ERG* spatial gene proximity

To confirm the role of AR in the dynamic reorganization of chromosomes, we investigated the effect of AR stimulation on *TMPRSS2* and *ERG* gene proximity. Using FISH we observed that, following DHT stimulation (3 hours), *TMPRSS2* and *ERG* co-localization was significantly increased in LNCaP, PNT1a and PNT2 cells (9.9 to 17.6%, $p<0.01$, 12.6 to 23.5%, $p<0.01$, and 12.7 to 19.8%, $p<0.05$, respectively) (Fig. 3 and Supplementary Table S2). However, AR-negative DU145 cells showed no increase in *TMPRSS2* and *ERG* co-localization (Fig. 3 and Supplementary Table S2). These observations are consistent with recent publications(6, 14) and confirm that DHT-stimulated gene fusions are associated with induced gene proximity through the AR pathway. PNT1a, PNT2 and LNCaP cells showed an inverse correlation of *AR* CAG repeat length ($n=20$, 19 and 25 respectively for PNT1a, PNT2 and LNCaP) with the rate of DHT induced *TMPRSS2* and *ERG* proximity, although this correlation needs to be confirmed in a larger sample set. Given our observation that *TMPRSS2:ERG* is more readily induced in cancer cells than non-malignant cells, it is interesting to find that the induced frequency of *TMPRSS2* and *ERG* co-localization is lower in LNCaP cells (17.6%) than PNT1a and PNT2 (23.5% and 19.8%, respectively) (Fig.

3). This suggests that cells with additional oncogenic abnormalities may be more susceptible to chromosome rearrangements than non-malignant cells.

***TMPRSS2:ERG* gene fusion is associated with *PIWIL1* expression**

The observation that gene co-localization and fusion frequencies are not directly correlated, suggests that the fusion rate is also dependent on other factors. In a recent study, the expression of *PIWIL1* was reported to be reduced in LNCaP cells(6). We investigated *PIWIL1* expression in our samples and found low *PIWIL1* expression in LNCaP and high expression in PrEC cells (Fig. 4). Interestingly, an intermediate level of expression was observed in PNT1a and PNT2 and expression in the fusion-positive VCaP cells could not be detected using the same PCR conditions. This suggests that *PIWIL1* expression is inversely correlated with the ability to induce *TMPRSS2:ERG* fusion and also indicates that PNT1a and PNT2 are better protected against chromosome rearrangements than LNCaP cells. Interestingly, in PNT1a and PNT2 cells *PIWIL1* expression was reduced following long-term DHT exposure (Fig. 4A). When considering *PIWIL1* expression (Fig. 4A) in combination with the frequency of induced gene proximity and gene fusion (Fig. 1 and 3) for each cell line, it appears that the prevention of DSBs by *PIWIL1* may be the main determinant in fusion frequency. Over-expression of *PIWIL1* in LNCaP cells was sufficient to prevent DHT-induced *TMPRSS2:ERG* fusion (Fig. 4B and C).

In conclusion, this study demonstrates that *TMPRSS2:ERG* fusion is an early event in prostate carcinogenesis and can be induced by androgen in a dose-dependent manner. The incidence of *TMPRSS2:ERG* events is associated with androgen-induced gene proximity as well as the reduced expression of genes, such as *PIWIL1*, which encode proteins that help protect the genome from DNA damage. The genetic factors, shown here to contribute to the incidence of *TMPRSS2:ERG* induction, may be applicable to the induction of other gene fusion events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank L. Xue, I. Bisson, A. Curtotti and C. Mein for technical support. We also appreciate the critical review and comments by Profs. B.D. Young and I. Hart.

Grant support: Orchid and UK Medical Research Council.

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Figure 1.

TMPRSS2:ERG can be induced in non-malignant prostate epithelial cells following long-term DHT treatment. *A*, Nested RT-PCR amplification of induced *TMPRSS2:ERG* fusion product in non-malignant prostate cell lines PNT1a and PNT2 following 5 months treatment with 300 and 3000 nM DHT. *B*, Automated DNA sequencing of RT-PCR products confirms *TMPRSS2:ERG* fusion (exon1:exon4). A representative image from PNT1a cells is shown. *C*, Schematic representation of the *TMPRSS2:ERG* fusion event.

Figure 2.

The *TMPRSS2:ERG* fusion cannot be detected in non-malignant prostate cell lines PNT1a and PNT2 following short-term DHT treatment. *A*, Nested RT-PCR was used to detect *TMPRSS2:ERG* fusion transcripts in the prostate cancer cell line LNCaP following 24 hours DHT treatment. No fusion product was detected in PNT1a, PNT2 and DU145 cells. *B*, Automated DNA sequencing of fusion-positive RT-PCR product confirm *TMPRSS2:ERG* fusion (exon1:exon4) in DHT treated LNCaP cells.

Figure 3. *TMPRSS2* and *ERG* gene proximity was induced in non-malignant prostate cell lines PNT1a and PNT2 following DHT treatment. **A**, *TMPRSS2* (red) and *ERG* (green) gene proximity was assessed by FISH in cells treated with and without DHT. * $p < 0.05$; ** $p < 0.01$. **B**, Representative FISH images.

Figure 4.

TMPRSS2:ERG gene fusion frequency is associated with *PIWIL1* expression. **A**, *PIWIL1* expression, detected by Q-RT-PCR, was reduced in LNCaP as compared to PrEC, PNT1a and PNT2 cells and was undetectable in VCaP cells. **B**, *PIWIL1* mRNA levels in LNCaP cells transfected with *PIWIL1* expression construct. **C**, Nested RT-PCR detection of the *TMPRSS2:ERG* fusion transcript in LNCaP over-expressing *PIWIL1*. No fusion product was detected in *PIWIL1* transfected cells. Ctrl: untreated control cells; 300 nM: cells with long-term DHT treatment at 300 nM; 3000 nM: cells with long-term DHT treatment at 3000 nM; *PIWIL1*: *PIWIL1* transfected cells; GFP: GFP transfected cells; DHT: 24-hour DHT treated cells.