Androgen-Induced TMPRSS2:ERG Fusion in Nonmalignant 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, could 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 nonmalignant prostate epithelial cells. Although the fusion could be detected in malignant cells following 24-hour treatment, prolonged exposure to androgen was required to detect the fusion transcript in nonmalignant 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. Cancer Res; 70(23); 1–5. ©2010 AACR.

Introduction

Gene fusion events are a hallmark of human hematologic 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 TMPRSS2:ERG fusion, detected in approximately 50% of prostate cancers, is the most common fusion gene found in human malignances (2, 4). This fusion results in the overexpression 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 nonmalignant 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 the 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 patient consent.
Dihydrotestosterone treatment

For colocalization analysis following short-term dihydrotestosterone (DHT) treatment, cells were cultured in RPMI medium with 10% carbon-stripped serum (Autogen Bioclear) for 24 hours. Cells were either treated for 3 hours with 100 nmol/L of DHT or cultured under the same conditions without DHT. For long-term treatment, cells were cultured in DMEM with 10% standard fetal bovine serum and additional 300 or 3,000 nmol/L of DHT. Medium was changed twice weekly and fresh DHT was added each time.

**AR microsatellite analysis**

AR CAG repeat length was determined by microsatellite analysis. PCR using primers (AR_FAM_F: 5'-FAM-ACCCA-GAGCCGCGAGCGCAG 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. Primer pairs TMPRSS2_F (5'-GGAGCCGCGAGCGCAG) and ERG_R (5'-CATATCTTCCACCCCATC, and AR_F (5'-ACCAGAGCCGCGAGCGCAG) 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 quantitative 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 the starting template.

**Fluorescence in situ hybridization**

TMPRSS2 and ERG colocalization analysis was performed using standard fluorescence in situ hybridization (FISH) protocol with 2 bacterial artificial chromosomes (BACs), RP11-3SC4 (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 the percentage of colocalized 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/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 5 foci were considered positive. A minimum of 100 cells were counted.

**Cell transfection**

LNCaP cells were transfected with PIWIL1 expression plasmid (Origene), using Nucleofector Kit B (Lonza) according to the manufacturer’s protocol.

**Statistical analysis**

Differences in the frequency of colocalization signals between groups were compared using a 2-tailed chi-square test. The values of P < 0.05 were considered statistically significant.

**Results and Discussion**

Androgen can induce TMPRSS2:ERG gene fusion in both malignant and nonmalignant 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 nonmalignant cell lines, PNT1a and PNT2, continuously with high doses (300 and 3,000 nmol/L) of the AR ligand DHT. After 5-month treatment, we detected the fusion transcript in both cell lines treated with 3,000 nmol/L of DHT and PNT2 cells treated with 300 nmol/L of 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 that induction is dosage associated. We treated cells with 100 nmol/L of DHT for 24 hours and detected fusion products confirming TMPRSS2:ERG fusion event.

![Image](http://cancerres.aacrjournals.org/).
transcripts in LNCaP cells but not in PrEC, PNT1a, and PNT2 cells (Fig. 2A and B). As the long pathogenesis of prostate cancer in vivo suggests that physiologic DHT doses would be unable to induce TMPRSS2:ERG in the short term, the doses used exceeded normal physiologic 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 cells but not in PrEC cells (6). This was confirmed by our short-term treatment of nonmalignant 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 premalignant 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. Although TMPRSS2:ERG has previously been observed in nonmalignant 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, suggest 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 whether 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 prostate cancer samples than in Western prostate cancer samples (19). Interestingly, compared with 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 colocalization was significantly increased in LNCaP, PNT1a, and PNT2 cells (9.9%–17.6%, $P < 0.01$; 12.6%–23.5%, $P < 0.01$; and 12.7%–19.8%, $P < 0.05$, respectively) (Fig. 3 and Supplementary Table S2). However, AR-negative DU145 cells showed no increase in TMPRSS2 and ERG colocalization (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 ($r = 20, 19,$ and 25 for PNT1a, PNT2, and LNCaP, respectively) with the rate of DHT-induced TMPRSS2 and ERG proximity, although this correlation needs to be confirmed in a larger sample size.
sample set. Given our observation that TMPRSS2:ERG is more readily induced in cancer cells than in nonmalignant cells, it is interesting to find that the induced frequency of TMPRSS2 and ERG colocalization is lower in LNCaP cells (17.6%) than in PNT1a and PNT2 cells (23.5% and 19.8%, respectively) (Fig. 3). This suggests that cells with additional oncogenic abnormalities may be more susceptible to chromosome rearrangements than nonmalignant cells.

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

The observation that gene colocalization 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 cells and high expression in PrEC cells (Fig. 4). Interestingly, an intermediate level of expression was observed in PNT1a and PNT2 cells 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 cells 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 (Figs. 1 and 3) for each cell line, it seems that the prevention of DSBs by PIWIL1 may be the main determinant in fusion frequency. Overexpression 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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