Reactivation of Androgen Receptor–Regulated TMPRSS2:ERG Gene Expression in Castration-Resistant Prostate Cancer

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Abstract

It seems clear that androgen receptor (AR)–regulated expression of the TMPRSS2:ERG fusion gene plays an early role in prostate cancer (PC) development or progression, but the extent to which TMPRSS2:ERG is down-regulated in response to androgen deprivation therapy (ADT) and whether AR reactivates TMPRSS2:ERG expression in castration-resistant PC (CRPC) have not been determined. We show that ERG message levels in TMPRSS2:ERG fusion-positive CRPC are comparable with the levels in fusion gene–positive primary PC, consistent with the conclusion that the TMPRSS2:ERG expression is reactivated by AR in CRPC. To further assess whether TMPRSS2:ERG expression is initially down-regulated in response to ADT, we examined VCaP cells, which express the TMPRSS2:ERG fusion gene, and xenografts. ERG message and protein rapidly declined in response to removal of androgen in vitro and in vivo. Moreover, as observed in the clinical samples, ERG expression was fully restored in the VCaP xenografts that relapsed after castration, coincident with AR reactivation. AR reactivation in the relapsed xenografts was also associated with marked increases in mRNA encoding AR and androgen synthetic enzymes. These results show that expression of TMPRSS2:ERG, similarly to other AR-regulated genes, is restored in CRPC and may contribute to tumor progression.

Introduction

A major breakthrough in prostate cancer (PC) was identification of recurrent fusions between androgen-regulated TMPRSS2 and Ets transcription factor genes (primarily ERG), placing the Ets genes under androgen-stimulated regulation of TMPRSS2 (1). Remarkably, this fusion is in preneoplastic lesions and ~50% of primary PC, consistent with an early role in tumor development (1–6). The standard treatment for locally recurrent/metastatic PC is androgen deprivation therapy (ADT), but patients invariably relapse with more aggressive tumors termed castration-resistant PC (CRPC). Significantly, androgen receptor (AR) is expressed at high levels in CRPC, as are multiple AR-regulated genes, indicating that AR transcriptional activity is at least partially reactivated (7, 8). Mechanisms contributing to this reactivation include increased intratumoral androgen accumulation/synthesis (8–12), AR overexpression, AR mutations (in AR antagonist–treated patients), and activation of kinase pathways that enhance AR activity.

TMPRSS2 is decreased in response to ADT (13), and it is presumed that TMPRSS2:ERG expression would also be decreased, which may contribute to responses, but this has not been shown directly in patients. The extent to which the TMPRSS2:ERG gene is expressed in CRPC and contributes to relapse is also unclear. One study of CRPC with the TMPRSS2:ERG gene found that it was not expressed, but this was in atypical AR-negative tumors (6). In contrast, the initial identification of fusion gene transcripts included CRPC tumors, although these were a small subset of outliers expressing very high ERG message levels (1). Therefore, to determine the extent to which TMPRSS2:ERG gene expression is reactivated in CRPC, we examined ERG expression in TMPRSS2:ERG fusion-positive primary androgen-dependent PC and CRPC clinical samples and in VCaP xenografts (14) before and after castration.

Materials and Methods

Cell culture and xenografts. Cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). For DHT treatment, cells were first grown to 50% to 60% confluence in 5% charcoal/dextran-stripped FBS (CSS) medium for 3 d. VCaP xenografts were established in the flanks of male severe combined immunodeficient mice by injecting ~2 million cells in 50% Matrigel. When tumors reached ~1 cm, biopsies were obtained and the mice were castrated. Additional biopsies were obtained 4 d after castration, and the tumors were harvested at relapse. Frozen sections confirmed that samples contained predominantly nonnecrotic tumor.

Reverse transcription-PCR and immunoblotting. Real-time reverse transcription-PCR (RT-PCR) used 50 ng RNA, and the results were normalized by coamplification of 18S RNA (see Supplementary Data). Blots were incubated with anti-ERG (1:1,000, polyclonal; Santa Cruz Biotechnology), anti–prostate-specific antigen (PSA; 1:3,000, polyclonal; BioDesign), anti-AR (1:2,000, polyclonal; Upstate), anti-p-AR(Ser81) (1:1,000, polyclonal; Upstate), or anti-actin (1:5,000, monoclonal; Abcam) and then with secondary antibodies (Promega).

Immunohistochemistry. Paraffin sections were boiled for 30 min in 10 mmol/L citrate buffer (pH 6.2) and blocked using 5% goat serum and avidin blocking solution (Vector). Primary antibodies, anti-AR (1:50) or anti-ERG (1:200), were added overnight at 4°C followed by biotinylated goat anti-rabbit antibody (1:800) and streptavidin-horseradish peroxidase (1:400; Vector). Slides were developed with 3,3′-diaminobenzidine and counterstained with hematoxylin.

Results and Discussion

ERG is expressed at comparable levels in TMPRSS2:ERG–positive primary PC and CRPC. Using RT-PCR on RNA from previously described CRPC bone marrow metastases (8), we detected TMPRSS2:ERG transcripts (TMPRSS2 exon 2-ERG exon 4) in 11 of 29 cases. Affymetrix oligonucleotide microarray data on these tumors versus a group of 27 microdissected primary PC (from the same study) were then examined for ERG expression.
Although the fusion status of the latter primary tumors was not known (RNA and tumor tissues were no longer available), ERG expression distinguished two nonoverlapping groups that presumably reflected fusion-negative (13 of 27) and fusion-positive tumors (14 of 27; Fig. 1A, AD, left). The majority of the fusion-negative CRPC had low ERG levels that were comparable with the levels in the ERG low primary PC group (Fig. 1A, AD, left). However, ERG expression in six of the fusion-negative CRPC was higher (Fig. 1A, circled), suggesting TMPRSS2:ERG fusions that were not picked up by RT-PCR. These six samples also all had low TMPRSS2 mRNA levels, suggesting a TMPRSS2:ERG fusion and loss of TMPRSS2 expression from one allele (Fig. 1A, left, circled). The panels on the right of Fig. 1A show these six tumors reclassified as fusion positive, which would indicate that ERG is expressed at comparable low levels in the fusion-negative primary (androgen dependent) and castration-resistant (androgen independent) tumors. Importantly, independent of how these six tumors are classified, ERG expression in the fusion-positive CRPC was significantly higher than in the fusion-negative CRPC. Moreover, ERG expression in the fusion-positive androgen-dependent and androgen-independent tumors was comparable based on overlapping confidence intervals, with a

Figure 1. ERG expression in TMPRSS2:ERG-positive primary PC and CRPC. A and B, expression in fusion-negative versus fusion-positive primary PC [androgen-dependent (AD)] and CRPC [androgen-independent (AI)]. C, ERG and TMPRSS2 expression by RT-PCR in these 29 CRPC tumors (AI) versus another group of 10 untreated primary PC (AD) and 6 normal prostates (N). Brackets, 95% confidence intervals for fusion-positive tumors. P values for differences between fusion-negative and fusion-positive androgen-dependent or androgen-independent tumors are shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
>95% probability that levels in androgen independent tumors are at least 50% of those in androgen-dependent tumors (Fig. 1A).

We reported previously that AR mRNA was consistently increased in CRPC and that multiple AR-regulated genes were highly expressed (8). As shown in Fig. 1B, expression of AR-regulated and androgen-regulated genes was similarly increased in the fusion-positive versus fusion-negative CRPC, consistent with comparable AR reactivation in these tumors. Interestingly, whereas expression of most AR-regulated genes was 2- to 3-fold lower in CRPC, ERG expression seemed to be more fully restored, suggesting that factors in addition to AR may be further enhancing TMPRSS2:ERG expression in CRPC.

To confirm these results, we examined ERG expression by real-time RT-PCR in the CRPC samples and in another independent small set of fusion-negative and fusion-positive primary PC. Significantly, this analysis also showed that ERG expression in the fusion-positive CRPC samples was increased ~4-fold compared with the fusion-negative CRPC and was comparable with expression in the fusion-positive primary PC (Fig. 1C). Taken together, these results show that the TMPRSS2:ERG fusion gene is expressed in CRPC at levels that are comparable with those in untreated primary PC, which presumably reflects at least in part reactivation of AR.

**Expressions of TMPRSS2:ERG transcript and ERG protein are androgen stimulated in VCaP cells.** Although the above data establish that TMPRSS2:ERG is comparably expressed in primary PC and CRPC, we have not yet been able to directly follow fusion gene expression in vivo in patients during ADT. Therefore, we next examined VCaP cells, which express AR and the common TMPRSS2:ERG fusion gene (14). Expression of an ~50 kDa protein, consistent with NH2-terminal truncated ERG, could be induced rapidly and at low DHT levels (0.1 nmol/L) in VCaP but not fusion-negative LNCaP cells (Fig. 2A). ERG, PSA, and TMPRSS2 (from the intact allele) mRNA were similarly induced in VCaP (Fig. 2B). Interestingly, induction was 2-fold higher for TMPRSS2 than ERG, suggesting that additional proteins may be increasing basal TMPRSS2:ERG expression. An AR antagonist, bicalutamide, suppressed DHT-stimulated expression of ERG (Fig. 2C). Taken together, these observations indicate that AR is similarly regulating both the wild-type TMPRSS2 and the TMPRSS2:ERG fusion gene.

![Figure 2. Androgen-regulated TMPRSS2:ERG expression in VCaP. A, cells in CSS medium were treated with DHT and immunoblotted. B, RT-PCR for ERG (exon 9/10), TMPRSS2 (exon 5/6), and PSA mRNA after DHT stimulation. C, cells in CSS medium treated with DHT and bicalutamide (Bic) for 24 h. D, cells in 5% CSS for 3 d were transfected with 10 nmol/L control or ERG siRNA (Dharmacon). DHT was added 8 h after transfection and cells were assayed for ERG protein and cell recovery (by MTT assay) after 3 d (with comparable results at 5 d).](image-url)
Finally, using small interfering RNA (siRNA) to decrease ERG expression, we did not observe marked effects on cell growth in the presence or absence of DHT (Fig. 2).

**TMPRSS2:ERG expression in VCaP xenografts is ablated by castration and reactivated in relapsed tumors.** S.c. VCaP xenografts were biopsied before castration, at 4 days after castration, and at ~6 weeks when tumors were growing rapidly and reached ~1 cm (Fig. 3A). As expected, PSA mRNA was decreased at 4 days and restored in the relapsed tumors (Fig. 3B). Moreover, ERG and TMPRSS2 expressions were also markedly decreased after castration and returned to precastration levels in the relapsed tumors, consistent with AR
reactivation (Fig. 3B). Although the importance of ERG expression in vitro remains unclear, previous data suggest that ERG functions in vivo by inducing genes that enhance tumor invasion, including matrix metalloproteinases (MMP) that are established Ets target genes (15–17). Significantly, MMP3 expression markedly declined after castration and returned to at least precastration levels in the relapsed tumors, supporting a role for ERG in recurrence.

Figure 4. Androgen synthetic enzymes in VCaP xenografts. A, enzymes in androgen metabolism. B, relative expression of indicated enzymes by RT-PCR in VCaP xenografts (before castration, blank columns; 4 d after castration, gray columns; relapse, black columns). C, VCaP cells in CSS medium were treated with 0, 0.1, 1, or 10 nmol/L of DHT for 24 h and assessed by RT-PCR for the indicated transcripts. D, VCaP cells grown in CSS medium were treated with (left) 0, 0.1, 1, or 10 nmol/L of DHT minus or plus bicalutamide (10 μmol/L) for 24 h, or (right) cycloheximide (CHX; 10 ng/mL) minus or plus DHT (10 nmol/L) for 0 to 8 h. Actinomycin D and cycloheximide were added 1 h before DHT. Results were normalized to 18S RNA.
Previous analyses of CRPC clinical samples and xenograft models have shown that AR mRNA is highly expressed and increased compared with levels before androgen deprivation (7, 8, 18, 19). Consistent with these data, AR mRNA in VCaP xenografts was increased after castration and was further increased in the relapsed tumors (Fig. 3D). In contrast, AR protein was markedly decreased at 4 days but was increased in the relapsed tumors (Fig. 3C). Nuclear ERG and AR expression were observed by immunohistochemistry in the VCaP xenografts before castration (Fig. 3D). Both declined markedly 4 days after castration, and the remaining AR at this time seemed to be cytoplasmic. Consistent with the RT-PCR and immunoblotting results, immunohistochemistry showed that both ERG and AR expressions were restored in the relapsed tumors. Whereas AR expression was primarily nuclear before castration, intense nuclear and diffuse cytoplasmic AR expressions were observed in the relapsed tumors. Taken together, these data show that TMPRSS2:ERG expression is markedly decreased in response to castration and that expression is restored in conjunction with AR reactivation in relapsed tumors.

**Relapsed VCaP xenografts have increased expression of enzymes mediating androgen synthesis.** PC may adapt to ADT by enhancing synthesis of androgens from weak steroid precursors and/or by de novo synthesis (8, 11, 12). Therefore, we assessed androgen synthetic enzymes before and after castration (Fig. 4A). ARK1C3 was increased after 4 days and went up further in the relapsed tumors (Fig. 4B). ARK1C3 expression in VCaP cells *in vitro* was also rapidly decreased by DHT, indicating that it is negatively regulated by androgen (Fig. 4C). Further experiments showed that this negative regulation was agonist dependent (blocked by an AR antagonist, bicalutamide) and reflected decreased transcription rather than increased mRNA degradation as it was not observed in actinomycin D-treated cells (Fig. 4D). Finally, DHT decreased ARK1C3 mRNA in cells treated with cycloheximide, indicating that new protein synthesis was not required and suggesting that the agonist liganded AR may be directly suppressing ARK1C3 transcription.

HSDB17B3 in testes carries out the same reaction as ARK1C3 but not normally in prostate. Although it was increased by castration, its basal expression was at the lower limits of detection. HSDB17B6, which regenerates DHT from its major metabolite (3α-diol; ref. 20), was also increased in the relapsed tumors (Fig. 4B) and was rapidly down-regulated by DHT *in vitro* (Fig. 4C). CYP17A1 and HSDB2 were increased after castration but returned to approximately baseline levels in the relapsed tumors (it should be noted that rodent adrenal glands do not express CYP17, so serum levels of "adrenal androgens" are low in castrate mice). These observations indicate that the entire steroidogenesis pathway is up-regulated acutely in response to ADT in VCaP xenografts and that further up-regulation of ARK1C3 and HSDB17B6, in conjunction with increased AR, may eventually provide the tumors with adequate androgen for AR reactivation and expression of AR-regulated genes, including TMPRSS2:ERG.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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