Androgens Modulate Expression of Transcription Intermediary Factor 2, an Androgen Receptor Coactivator whose Expression Level Correlates with Early Biochemical Recurrence in Prostate Cancer


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Introduction

Prostate cancer is an androgen-dependent disease; metastatic prostate cancer is typically treated by androgen receptor (AR) blockade. Recurrence after androgen ablation and evidence that AR continues to play a role in many prostate cancers has led to an examination of other factors that potentiate AR activity. AR is a ligand-activated transcription factor whose activity is regulated not only by hormone but also by the levels of coactivators recruited by AR to facilitate transcription. We sought to assess the consequences of reducing expression of the transcription intermediary factor 2 (TIF2) coactivator on prostate cancer cell growth and AR action in cell lines to examine TIF2 expression in prostate cancer and to correlate expression with clinical outcome. Depletion of TIF2 reduced expression of AR-induced target genes and slowed proliferation of AR-dependent and AR-independent prostate cancer cells. Remarkably, we found that TIF2 expression is directly repressed by high levels of androgens in multiple AR-expressing cell lines. Expression of a reporter containing 5-flanking region of the TIF2 was repressed both by androgens and by the antagonist, Casodex. Expression of TIF2 correlates with biochemical (prostate-specific antigen) recurrence (P = 0.0136). In agreement with our in vitro findings, the highest expression of TIF2 was found in patients whose cancer relapsed after androgen ablation therapy, supporting the idea that AR blockade might activate pathways that lead to stimulation of AR-dependent and AR-independent proliferation of prostate epithelium. The elevated expression of TIF2 at low hormone levels likely aids in inducing AR activity under these conditions; treatment with Casodex has the potential to counteract this induction. (Cancer Res 2006; 66(21): 10594-602)

Abstract

Prostate cancer is an androgen-dependent disease; metastatic prostate cancer is typically treated by androgen receptor (AR) blockade. Recurrence after androgen ablation and evidence that AR continues to play a role in many prostate cancers has led to an examination of other factors that potentiate AR activity. AR is a ligand-activated transcription factor whose activity is regulated not only by hormone but also by the levels of coactivators recruited by AR to facilitate transcription. We sought to assess the consequences of reducing expression of the transcription intermediary factor 2 (TIF2) coactivator on prostate cancer cell growth and AR action in cell lines to examine TIF2 expression in prostate cancer and to correlate expression with clinical outcome. Depletion of TIF2 reduced expression of AR-induced target genes and slowed proliferation of AR-dependent and AR-independent prostate cancer cells. Remarkably, we found that TIF2 expression is directly repressed by high levels of androgens in multiple AR-expressing cell lines. Expression of a reporter containing 5-flanking region of the TIF2 was repressed both by androgens and by the antagonist, Casodex. Expression of TIF2 correlates with biochemical (prostate-specific antigen) recurrence (P = 0.0136). In agreement with our in vitro findings, the highest expression of TIF2 was found in patients whose cancer relapsed after androgen ablation therapy, supporting the idea that AR blockade might activate pathways that lead to stimulation of AR-dependent and AR-independent proliferation of prostate epithelium. The elevated expression of TIF2 at low hormone levels likely aids in inducing AR activity under these conditions; treatment with Casodex has the potential to counteract this induction. (Cancer Res 2006; 66(21): 10594-602)

Introduction

Because prostate cancer is an androgen-dependent disease, metastatic prostate cancer is treated by androgen receptor (AR) blockade. Rarely curative, tumors resistant to therapy develop and are detected by rising serum levels of prostate-specific antigen (PSA), an androgen-regulated protease. Studies using in vitro models, animal models, and human tumors suggest that AR plays a key role in androgen-independent tumors. Studies using AR small interfering RNA (siRNA) or AR antibodies have shown that androgen-independent prostate cancer cell lines can retain AR dependence (1, 2). Increased AR expression in androgen-independent tumors relative to androgen-dependent tumors is consistently observed both in xenograft models (3) and in human tumors (4). These findings have led to renewed interest in understanding the regulation of AR action to develop alternate means to block its activity.

AR is a member of the nuclear receptor family of ligand-activated transcription factors (5, 6). On binding hormone, it binds to target DNA sequences recruiting coactivators that facilitate transcription. AR activity depends on recruitment of coactivators from the limited cellular pool. Overexpression of a key coactivator can enhance AR activity; reducing its expression and/or interaction with AR reduces activity. We found that higher steroid receptor coactivator (SRC)-1 expression in prostate cancer correlates with tumor aggressiveness and that reducing its expression in prostate cancer cell lines reduces AR-dependent proliferation and inhibits the ability of AR either to activate or repress target genes (2).

Transcription intermediary factor 2 (TIF2) is the member of the p160 family of coactivators that is most closely related to SRC-1 (2). TIF2 overexpression in cell culture enhances AR activity (7); a small study comparing androgen-independent tumors with androgen-dependent tumors and benign prostate hyperplasia samples indicated that TIF2 is likely overexpressed in androgen-independent compared with androgen-dependent prostate cancers (8). We sought to examine the expression of TIF2 in a large number of prostate tumors to correlate expression with clinical outcome and to determine the consequences of reducing TIF2 expression on prostate cancer cell proliferation and AR action. These studies not only showed that TIF2 is required for optimal prostate cancer cell proliferation and that its expression correlates with aggressive disease but also uncovered a novel feedback regulatory mechanism, in which ligand-bound AR directly represses TIF2 expression. Reducing androgen levels elevates TIF2 expression, presumably facilitating AR action at suboptimal levels of androgens.

Materials and Methods

Cell culture. Androgen-dependent LNCaP prostate cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 containing 10% fetal bovine serum (FBS; Intergen Co., Purchase, NY) with penicillin and streptomycin (Invitrogen, Carlsbad, CA). AR-negative PC-3 prostate cancer cells were from ATCC and maintained in DMEM/F12-10% FBS and penicillin/streptomycin. The
was carried out as follows: a 3-minute hot start at 95°C, cycles of denaturation at 95°C for 30 seconds, and an extension at 72°C for 30 seconds. Each experiment was done in triplicate.

Materials. [3H]Thymidine was purchased from ICN (Irvine, CA). Rabbit anti-mouse IgG was from Zymed Laboratories, Inc. (San Francisco, CA). R881 was from Perkin-Elmer Life Sciences (Boston, MA). Ru486 was from EMD Biosciences (San Diego, CA), and Cosadem was from LKT Laboratories (St. Paul, MN). Horseradish peroxidase–conjugated anti-rabbit IgG and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The TIF2-luciferase and TIF2-luciferase reporter constructs were generated using plasmid DNA. Threshold values were used for quantification and expressed as copy number.

A 300 ng/mL at 4°C. Anti-TIF2 mouse monoclonal antibody (BD Biosciences, San Jose, CA) was used at a 1:500, or an actin antibody (Chemicon International, Temecula, CA) at a 1:1,000 dilution and ECL reagents. slides were scanned using the Bliss automated slide scanner system to produce high-resolution digital images. Staining was evaluated in normal luminal epithelial nuclei in normal samples or in the epithelial tumor cell nuclei of prostate cancer samples as described (15). Staining intensity was graded as absent (0), weak (1+), intermediate (2+), or strong (3+). Staining extent was scored as follows: no staining (0), 1% to 25% of cells stained (1+), 26% to 50% of cells stained (2+), or 51% to 100% of cells stained (3+). The staining index for each case was then calculated by multiplying the average intensity score for the three cores by the average percentage score for the three cores, yielding a 10-point staining index ranging from 0 (no staining) to 9 (extensive, strong staining). Sections from six tissue microarrays of prostate cancer were provided by the Baylor Prostate Cancer Specialized Programs of Research Excellence (SPORE) tissue bank. Total RNA was extracted from Trizol reagent (Invitrogen); cDNA synthesis was done using an Invitrogen SuperScript first-strand synthesis system. Primers for TIF2 were as follows: GCTGCCAACATAGATGAAGTG (forward) and CATTCTCGATCACAACAAACACA (reverse). Primers for keratin 18 were as follows: AGGGTCTGACTTCTCGAAAT (forward) and GTATCAATGACTCTGGGAGGAG (reverse). Real-time PCR was carried out as described previously (14).

The amplification protocol for TIF2 was as follows: 1.5-minute hot start at 95°C for 40 cycles of denaturation at 95°C for 30 seconds and annealing at 70°C for 30 seconds, with a final cycle of annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. The keratin 18 protocol was carried out as follows: a 3-minute hot start at 95°C followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 20 seconds, and a 72°C extension for 30 seconds. Each experiment was done in duplicate. The Ct values in log-linear range representing the detection threshold values were used for quantification and expressed as copy numbers based on a standard curve generated using plasmid DNA.

Tissue microarrays and immunohistochemistry. The tissue microarrays have been described (15). Briefly, three 0.6-mm cores of cancer and normal peripheral zone tissue were obtained from radical prostatectomy specimens and used to construct tissue microarrays. Patients received no chemotherapy or androgen ablation therapy. Other patient characteristics were described previously (15). Immunohistochemical analyses were done as described (16). Briefly, antigen retrieval was done in six-well plates in medium containing FBS, fixed in 4% formaldehyde, and incorporated [3H]thyminidine was determined (18).

Western blot analysis. Transfected cells were lysed in TES buffer (10 mM Tris, 1 mM EDTA, 12 mM thioglycolate (pH 7.7)) by three rounds of freeze/thaw; 1 to 10 μg protein (PSA) or 20 to 30 μg (TIF2) were resolved on 12.5% or 6.5% SDS-PAGE, respectively. Proteins were detected as described (2) using either a PSA antibody (DAKO, Carpinteria, CA) at a 1:2,000 dilution, TIF2 antibody (BD Biosciences) at a dilution of 1:500, or an actin antibody (Chemicon International, Temecula, CA) at a 1:1,000 dilution and ECL reagents.

Immunofluorescence. LNCaP and PC-3 cells were grown on coverslips in six-well plates in medium containing FBS, fixed in 4% formaldehyde, and incubated with anti-TIF2 antibody at a concentration 1:500 (BD Biosciences) followed by FITC-conjugated goat anti-mouse IgM (Zymed). The coverslips were mounted in Vectashield mounting medium containing 4'-6-diamidino-2-phenylindole (Vector Laboratories) and fluorescence was detected using a Zeiss (Thornwood, NY) AxioPlan2 microscope. The nominal exposure was found for detection of the fluorescent signal in cON-treated cells. Fluorescence in cells treated with TIF2 asON was captured with the same exposure times as controls and left unscaled using MetaView software.

Preparation of RNA from cells and real-time quantitative PCR. RNA was extracted using Trizol reagent and dissolved in 60 μl diethylpyrocarbonate-treated water. RNA solution (2-10 μl) was used as a template for real-time quantitative reverse transcription-PCR (RT-PCR) using Taqman One-Step RT-PCR Master Mix reagents. The PCR was run on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Data were normalized to 18S rRNA expression. To measure 18S expression, the primary RNA stock was diluted 1,000-fold and 5 μl were used in a RT-PCR using Taqman rRNA Control and One-Step RT-PCR Master Mix Reagents (Applied Biosystems). Each point was done in triplicate and the average and SD were calculated. Maspin detection was described previously (2).

The primers used as follows: TIF2, CGTGCTCTAGCTGAGGCT, CTTCCCCTCAGAGGAGGATCA, and FM-CCTTCCATGTGGCCCAGGAGG-TMARA (Applied Biosystems); SH00P ampiclon, GCTGTAGGAGAAGGAGTCACCA, FM-TGCAGAGTGGAAAGACAGAAGTGGCAG-TMARA, and GTGACATCTCAGGAAAGATATAT; PMEPA1, GTGATCACGTGGCAGGAGGAGGAGG; and S100P amplicon, GCTGATGGAGAAGGAGCTCTGCTAA-TMARA.
TGCTGTGCCGGCTG; TMPRSS, AGAATCGGTGTGTTCGCCTC, FAM-
ACCAAACTTCATCCTCCAGGTGACTCATCTCAGAG-TAMRA, and
CTCGTTCCAGTCGTCTTGGC; and PSA, ACCTGCACCCGGAGAGCT, FAM-
TGTCACCATGTGGGTCCCGGTTG-TAMRA, and TCACGGACAGGGTGAG-
GAAG.

Chromatin immunoprecipitation assay. LNCaP cells were incubated in medium supplemented with sFBS for 48 hours, incubated with

10 nmol/L R1881, cross-linked with formaldehyde, and harvested at the indicated time points. Cells were sonicated, and extracts were used for immunoprecipitation (19) with the AR-specific antibody, AR441 (13). DNA was recovered using a Qiagen kit (Valencia, CA) for purification of PCR DNA fragments. The PSA enhancer, TIF2 promoter, TIF2 intron, and control sequence from within the human serum- and glucocorticoid-induced kinase (SGK) gene were amplified with the following primers and probes: TIF2 promoter, TTTTGTCCCCTGTCCCCTTACT, CTCCGTCGGCA-GAGTCCTC, and FAM-TTCTTCACCTCTCCCCGGGCCG-TAMRA; TIF2 intron, TTGCTGAATGCATAAGGTATAAACTGTTA, TTGACTCAGTGCC-CATTACTTAGTTCT, and FAM-TCTCAGTATAAACATAGATTGTCCTC-
CAAGGACAGTGC-TAMRA; SGK gene, TGACCCCGAGTTTACCGAAG,
CCTTGACGCTGGCTGTGAC, and FAM-CCTGTCCCCAACTCCATTGG-
CAAGT-TAMRA (probe); and PSA enhancer, GCCCCCCCCAAATCTTGTA,
CCATCACAGACGGCATGATG, and FAM-TGACCAGAGCAGTCTAGGTG-
GATGCTGTG-TAMRA.

Immunoprecipitation of the AR-bound genomic sequences. Two flasks with 10 million LNCaP cells were cultured for 48 hours in medium supplemented with sFBS. Cells were treated with vehicle or 30 nmol/L R1881 for 2 hours, cross-linked, and harvested according to the GENpathway protocol (San Diego, CA). Immunoprecipitation and sequencing of AR-interacting genomic sequences was done by GENpathway.

Statistical analysis. Correlations between biomarkers and clinical and pathologic variables were evaluated using the Spearman correlation. The predictive value of TIF2 expression univariately and multivariately with other clinical and pathologic variables was analyzed using the Cox proportional hazards regression model and the hazard ratio (HR) and 95% confidence intervals (95% CI) were computed. Kaplan-Meier survival curves for different levels of TIF2 and TIF2/AR combinations were also plotted. Comparisons of mean levels of expression of specific mRNAs and transcriptional activity of AR on the transfected reporters were done using independent samples t tests and shown with bar charts, unless the normality assumptions about the data are violated, in which case the Mann-Whitney U test was used and box plots were used for illustration. P values < 0.05 were considered statistically significant for all tests. Analyses were done using the SPSS version 12.0 software package (SPSS, Inc., Chicago, IL).
**Results**

TIF2 is required for optimal cell growth and agonist-dependent AR target gene induction in LNCaP cells. To determine whether TIF2 is required for LNCaP cell growth and AR-dependent gene regulation, we used asON or siRNA to reduce TIF2 expression. Both reduced TIF2 mRNA (Fig. 1A) or protein (Supplementary Fig. S1) expression relative to the corresponding ON or control siRNA (Fig. 1A) and [3H]thymidine incorporation, a measure of cell proliferation (Fig. 1B). Expression of PSA protein (Fig. 1C) and mRNA (Fig. 1D), a prototypical androgen-regulated target, was also reduced. We found that down-regulation of SRC-1 expression was observed when TIF2 was depleted (Fig. 1D). However, TIF2 is required for optimal expression of genes positively regulated by AR, a Ca2+-binding protein, S100P (20), TMPRSS2 (an androgen-regulated protease whose androgen-responsive promoter region is often found fused to the coding region of an ETS factor in prostate cancers; ref. 21), and PMEPA1 (subunit of a cohesion complex and a mediator of p53-dependent apoptosis; refs. 22, 23; Fig. 1D).

**TIF2 is required for optimal proliferation of AR-negative PC-3 prostate cancer cells.** SRC-1 ablation reduced proliferation of LNCaP cells but not of the AR-negative PC-3 or DU145 cell lines, suggesting that the actions of SRC-1 were primarily AR dependent (2). However, TIF2 ablation (Fig. 2A) reduced [3H]thymidine incorporation in PC-3 cells (Fig. 2B); thus, TIF2 also affects proliferation independent of AR.

**TIF2 expression is down-regulated by androgens.** In carrying out the TIF2 ablation studies, we detected reduced TIF2 expression in cells grown in FBS rather than sFBS. Because a major difference between the two conditions is the lack of small hydrophobic molecules, including androgens in sFBS, we asked whether androgens repress TIF2; both TIF2 RNA and protein levels were reduced by R1881 treatment and levels were also lower in FBS, which contains endogenous androgens, than in sFBS in LNCaP, C4-2, and LAPC-4 cells (Fig. 3A). TIF2 mRNA levels in LNCaP cells were reduced by R1881 in the presence of cycloheximide, suggesting primary regulation by AR (Fig. 3B). Incorporation of [3H]thymidine in LNCaP cells is stimulated by low concentrations of androgen, but not by higher concentrations (Fig. 3C), and in some cases, are growth inhibitory (24). Stimulatory concentrations of androgen (100 pmol) do not down-regulate TIF2 expression, whereas higher concentrations repress TIF2 expression (Fig. 3C).

Note that TIF2 expression in FBS is lower than would be expected simply from the level of androgen; whether this is due to...
independent regulatory factors or growth factor-dependent potentiating of AR activity is unknown. We compared the ability of TIF2 to potentiate AR activity at low and high R1881 levels and found that hormone-dependent activation is substantially lower at 10 pmol R1881 than at 3 nmol/L R1881 (Fig. 3D); overexpression of TIF2 elevates activity at 10 pmol R1881 to a level higher than that observed at 3 nmol/L R1881 without added TIF2, although the activity at 3 nmol/L R1881 is also potentiated. The fold induction of hormone-dependent activity achieved by overexpression of TIF2 is higher at 10 pmol/L R1881 than at 3 nmol/L R1881 (fold induction TIF2 at 10 pmol/L/fold induction at 3 nmol/L = 1.5 for an average of nine experiments). This suggests that the elevation in TIF2 levels when androgen levels are reduced aids in maintaining receptor activity.

AR is recruited to the region 5’ to the first exon of TIF2 as well as to a TIF2 intron sequence. In independent studies, we have been examining candidate AR-binding sites identified in a modified chromatin immunoprecipitation (ChIP) assay done commercially by GENpathway. Among the recovered sequences was one located in intron 8 of TIF2 (Fig. 4A). We used ChIP assays to determine whether AR is recruited to this intron and/or to the region immediately upstream of exon 1 and compared recruitment with recruitment to PSA, a characterized AR target we detected weaker but consistent recruitment to the promoter and to the intron, which kinetically mirrored recruitment of AR to the PSA enhancer (Fig. 4B). We did not detect ligand-dependent recruitment of AR to the irrelevant internal portion of the SGK gene (nucleotides 1212-1294 in the mRNA or 27-109 in exon 12; Fig. 4B).

Using Transcription Element Search Software, we analyzed a 2,000-bp region 5’ to exon 1 of TIF2 and found several clusters of potential AR-binding half sites in the 3’ portion of this region, but no consensus androgen response elements (ARE) containing two inverted half sites separated by three nucleotides. To determine if this region of the TIF2 promoter contributes to its down-regulation by androgens, we cloned 307 to -1 (TIF2) as well as a -540 to -1 fragment (TIF2L) into a pGL3 basic luciferase reporter (Fig. 5A) and examined its regulation by AR in transient transfection assays. Both pieces contained a conserved TATA box at -21 to -15. The activities of both promoters were repressed by agonist-bound AR (Fig. 5A) but the -357 to -1 fragment was sufficient to show AR-dependent inhibition. The basal activity of the longer promoter fragment was much lower than the shorter fragment (data not shown), suggesting that there is an additional negative regulatory element, but the percentage repression by R1881 is comparable for the two promoters. The regulation of the TIF2 reporter transfected into LNCaP cells paralleled the regulation of the endogenous gene (Fig. 5A, right). A previously characterized AR mutant (C619Y) that is incapable of binding DNA, but binds coactivators, including SRC-1 (13), does not repress TIF2 expression (Fig. 5B). Moreover, both agonist- and antagonist-bound receptor repressed the TIF2 reporter (Fig. 5C). Finally, AR with its ligand-binding domain (LBD) deleted activated the TIF2 reporter and reversed the repression by wild-type AR (Fig. 5D). Collectively, these studies suggest that direct DNA binding is required and that the AR LBD likely interferes with binding of a factor required for transcriptional activity.

Analysis of TIF2 expression in patient samples. To determine whether the requirement for TIF2 for growth of prostate cancer cells in vitro was reflected in elevated TIF2 expression in tumors, TIF2 mRNA and protein expression was measured. Quantitative real-time PCR was done on RNAs from 59 prostate cancers from men undergoing radical prostatectomy for clinically localized prostate cancer; 21 patients had no evidence of PSA recurrence after 5 years of follow-up, 16 patients had delayed PSA recurrence (mean time to recurrence, 33.6 months), 18 cancers recurred early [i.e., <1 year (mean time to recurrence, 3.5 months)], and 4 cancers were from patients with shorter follow-up not fitting into one of the above categories. Copy number of TIF2 mRNA was determined on reverse-transcribed RNAs and normalized to keratin-18 mRNA to control for variations in the percentage epithelial cells as TIF2 expression was reflected in elevated TIF2 expression in tumors, TIF2 mRNA and protein expression was measured. Quantitative real-time PCR was done on RNAs from 59 prostate cancers from men undergoing radical prostatectomy for clinically localized prostate cancer; 21 patients had no evidence of PSA recurrence after 5 years of follow-up, 16 patients had delayed PSA recurrence (mean time to recurrence, 33.6 months), 18 cancers recurred early [i.e., <1 year (mean time to recurrence, 3.5 months)], and 4 cancers were from patients with shorter follow-up not fitting into one of the above categories. Copy number of TIF2 mRNA was determined on reverse-transcribed RNAs and normalized to keratin-18 mRNA to control for variations in the percentage epithelial cells as TIF2 and keratin are expressed only in epithelial and cancer cells (see below). Relative levels of TIF2 expression were not significantly correlated with pathologic variables associated with aggressive disease. TIF2 expression was a significant predictor of time to recurrence as a continuous measure (P = 0.0351). The risk of experiencing a recurrence within individual follow-up was 2.39 times higher (95% CI, 1.16-4.94; P = 0.0186) in patients with high levels of TIF2 expression (i.e., the upper quartile; Fig. 6A). When the three groups were compared (Fig. 6B), patients that recurred during the 1st year of follow-up had significantly higher levels of TIF2 than those who had not recurred for at least 5 years after

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surgery (median levels, 0.0036 versus 0.0006; \( P = 0.0122 \)). Therefore, increased expression of TIF2 mRNA in clinically localized cancers is associated with early biochemical recurrence, an indicator of aggressive disease.

To extend these observations to the protein level on a larger group of patients, we did immunohistochemistry and analysis of TIF2 expression (Fig. 6C) on tissue arrays of clinically localized prostate cancers using an anti-TIF2 antibody as described in Materials and Methods. Staining was present almost exclusively in luminal epithelial cell and cancer cell nuclei. Interestingly, the normal epithelium had quite variable staining indices, ranging from 0 to 9. The staining indices of 518 cancers were determined. The cancers had quite variable staining indices (0-9; Fig. 6D). The three cores from each patient were highly consistent with each other so that this variability seemed to reflect differences between patients rather than heterogeneity within each specimen. There was a significant correlation of tumor TIF2 expression with the pathologic variables of cancer aggressiveness, such as Gleason score (\( P = 0.0267; \rho = 0.094 \)), seminal vesicle invasion (\( P = 0.0128; \rho = 0.109 \)), and pelvic lymph node metastasis (\( P = 0.0331; \rho = 0.094 \)). On univariate Cox regression analysis, there was a significant association of higher TIF2 levels with PSA recurrence (\( P = 0.0238 \)), although multivariately TIF2 staining intensity was not an independent predictor of biochemical recurrence (\( P = 0.0872 \)). Comparison of cancers with the highest TIF2 expression (staining index, \( 9; n = 181 \)) versus all other cancers (\( n = 308 \)) revealed that the cancers with the highest expression of TIF2 were 68% more likely to recur (HR, 1.68; 95% CI, 1.11-2.53; \( P = 0.0136 \)) as shown by the Kaplan-Meier plot in Fig. 6C. Because TIF2 is an AR coactivator, we used the previously reported results on AR expression in this array (25) to ask whether levels of AR in combination with levels of TIF2 were predictive of recurrence. Analysis of the relationship

\[ \text{Figure 5. TIF2 expression is repressed by agonist-bound AR.} \]

A, left, HeLa cells were transfected with 100 ng TIF2-luciferase, 30 ng pCR3.1 \( h \)-gal expression plasmid, and either 10 ng pCR3.1 vector or 10 ng pCR3.1 AR. After transfection, cells were treated with either vehicle or 3 nmol/L R1881 overnight and assayed for luciferase and \( h \)-gal activity. Middle, HeLa cells were transfected with 100 ng TIF2-luciferase reporter, 30 ng pCR3.1 \( h \)-gal expression plasmid, and either 10 ng pCR3.1 vector alone or 5 ng pCR3.1 AR and 10 ng pCR3.1 AR. After 3 hours, an equal volume of medium with 10% sFBS was added and cells were treated with 3 nmol/L R1881. The next day, cells were lysed and assayed for luciferase and \( h \)-gal activity. Right, LNCaP cells were transfected with 300 ng TIF2-luciferase reporter and 100 ng pCR3.1 \( h \)-gal per well in a six-well plate by electroporation and incubated in RPMI 1640 supplemented with 10% FBS, 10% sFBS, or 10% sFBS with 10 nmol/L R1881. Cells were lysed 48 hours later and assayed for luciferase and \( h \)-gal activity. Each point was done in triplicate.

B, HeLa cells were transfected with 100 ng TIF2-luciferase reporter, 30 ng \( h \)-gal expression plasmid, and either 5, 15, or 30 ng pCR3.1 AR. Cells were treated with vehicle (white column), 3 nmol/L R1881, or an AR antagonist either 1 nmol/L Casodex (Cas), 1 nmol/L hydroxyflutamide (HFl), 10 nmol/L RU486, or 100 nmol/L RU486 for 24 hours, then lysed, and assayed for luciferase and \( h \)-gal activity. The remaining lysate (4 \( \mu \)g) was resolved on SDS-PAGE and assayed for AR and actin levels by Western blotting.

D, HeLa cells were transfected with 100 ng TIF2-luciferase, 30 ng \( h \)-gal reporter plasmids, 15 ng pCR3.1 vector alone or with 5, 15, or 30 ng AR1-660, balanced with its expression plasmid to 30 ng (left, four white columns), or 15 ng pCR3.1-AR expression plasmid and 5, 15, or 30 ng AR1-660, balanced with its expression plasmid to 30 ng. Luciferase activity was normalized by the \( h \)-gal activity. All of the experiments were done thrice in triplicate and data were analyzed by t test. *, differences with \( P < 0.05 \).
between AR expression combined with TIF2 expression and time to recurrence, shown in Fig. 6C (right), revealed that patients with low AR (staining index, <3; n = 148) regardless of TIF2 levels had a slightly lower chance of recurrence than patients with high AR (staining index, ≥3) and low levels of TIF2 (HR, 1.94; 95% CI, 1.00-3.75; P = 0.0509; n = 177) and a significantly lower chance than those with high expression of both AR and TIF2 (HR, 3.12; 95% CI, 1.63-5.96; P = 0.0006; n = 148). There was a significant difference between those with high TIF2 versus low TIF2 among high AR patients (HR, 1.61; 95% CI, 1.01-2.57; P = 0.0437). Taken together, the results of the quantitative RT-PCR and tissue microarrays strongly indicate that prostate cancers with increased expression of TIF2 are significantly more aggressive than those with lower expression. We then asked whether TIF2 expression was associated with biological variables of increased aggressiveness—reduced apoptosis and increased proliferation that were previously measured using this array (26). TIF2 expression strongly correlated with increased tumor proliferation as determined by Ki-67 immunohistochemistry (P < 0.0001; r = 0.374). In addition TIF2 staining intensity was inversely correlated with apoptosis as determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (P < 0.0001; r = −0.259).

Immunohistochemical analysis of TIF2 expression in nine transurethral resections from men with recurrent, androgen-independent prostate cancer yielded a TIF2 staining index of 9.0 in all of these cases versus a median index of 6.0 (mean, 5.71; n = 518) in the radical prostatectomy patients, a statistically significant difference (P = 0.0010). Because the TIF2 staining indices were increased in cancers with higher Gleason score and eight of the nine recurrent androgen-independent cancers were poorly differentiated (Gleason scores 8-10), we compared TIF2 staining intensity in the 45 poorly differentiated cancers from radical
prostatectomies to that in these eight transurethral resections. The median staining index in the poorly differentiated primary cancers remained 6.0 (mean, 6.44; \( n = 45 \)) versus 9.0 in the recurrent cancers. This difference was statistically significant (\( P = 0.0109 \)). Thus, the strong expression of TIF2 in androgen-independent prostate cancers is independent of their higher Gleason scores and is consistent with our in vitro observation that reducing androgen levels increases TIF2 expression.

Discussion

Information about the role of AR coactivators in prostate cancer growth and androgen ablation resistance is limited. We found that TIF2 mRNA levels were significantly higher in tumors from men with early biochemical recurrence. Increased TIF2 protein was associated with multiple pathologic variables of aggressive disease, including increased Gleason score, seminal vesicle invasion, and pelvic lymph node metastasis, as well as PSA recurrence. Concordant with these clinical findings, TIF2 expression was associated with increased proliferation and decreased apoptosis. Reducing TIF2 expression in either AR-positive or AR-negative cells slowed proliferation, whereas SRC-1 regulated proliferation only in AR-dependent cell lines (2). The related coactivator, SRC-3 is also required for optimal proliferation of PC-3 cells (26). Our study raises the question of whether the requirement for TIF2 for optimal proliferation of AR-positive cells is an AR-dependent or AR-independent activity. Two findings argue that the requirement for TIF2 is at least in part AR dependent. TIF2 was required for optimal induction of some AR target genes. Thus, it clearly participates in AR-dependent actions. Second, the finding that high TIF2 expression is predictive of a shorter time to progression only when AR expression is high (Fig. 6C) suggests that it potentiates AR-dependent growth. Although TIF2 expression clearly is associated with aggressive clinical behavior in androgen-dependent, clinically localized prostate cancer, the relative contribution of AR-dependent and AR-independent actions to this aggressive behavior still needs to be elucidated.

Signaling pathways often have feedback loops regulating the extent of activation. We detected a reduction of TIF2 levels in the presence of androgens. Repression of TIF2 in independently derived AR-positive cell lines suggested that this is a general phenomenon. This was consistent with our finding that TIF2 expression was markedly higher in samples from patients following androgen ablation than in untreated tumors of comparable grade. Moreover, the finding that AR repression is cycloheximide insensitive suggests that TIF2 is a direct target of AR. Our demonstration that overexpression of TIF2 can in part compensate for reduced of androgens suggests that the up-regulation of TIF2 potentiates AR action under these conditions. Both androgen and Casodex inhibited expression from a reporter regulated by a fragment of the TIF2 promoter. Although this fragment does not contain a sequence identical to any of the previously reported AREs, it has multiple half sites and SP1-binding sites. Functional analyses using AR mutants, agonists, and antagonists suggest that the repression requires direct binding to the promoter and that the LBD is required. This also suggest a pathway for the emergence of androgen independence after androgen ablation: the initial success of inhibition of AR action may be undermined by the increase in TIF2 that ultimately contributes to androgen ablation resistant, but AR-dependent proliferation, as well as proliferation independent of AR. For TIF2, our promoter studies and our failure to reverse androgen repression of TIF2 levels by Casodex treatment (data not shown) suggest a combination of androgen ablation and Casodex would be beneficial in limiting AR activity and cell growth.

Although many candidate coactivators have been identified, little is known about their contributions to androgen-dependent regulation of endogenous target genes. We found previously that SRC-1 regulates both AR-activated and AR-repressed genes (2). In contrast, depletion of TIF2 reduced expression of the positively regulated AR target genes in LNCaP cells, whereas repression of maspin did not change. As we were preparing this article for publication, a report by Wang et al. (27) was published, indicating that depletion of TIF2 did not affect expression of PSA in LNCaP cells. Whereas we did our experiments in medium supplemented with full serum, Wang et al. (27) used a hormone-depleted medium that was likely depleted not only of androgens but also of growth factors. Apparently, a particular active cell signaling pathway is required for TIF2 to contribute to AR function as we found for SRC-1 and progesterone receptor (28) or that there is a difference in the LNCaP cells in the two laboratories. Gregory et al. (29) showed that reducing TIF2 expression reduces AR activity measured using a PSA-luc reporter in a CWR-R1 cell line showing a role for TIF2 in AR action in this independently derived cell line.

Our results indicate that TIF2 plays a role in promoting proliferation and aggressive clinical behavior in androgen-dependent prostate cancers and the emergence of androgen-independent tumors. The relative contribution of AR-dependent and AR-independent transcription in these processes still must be determined. These findings suggest that novel therapeutic approaches targeting steroid hormone coactivators may have broad clinical utility in the treatment of prostate cancer.

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References

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