Suppression of Prostate Tumor Growth by U19, a Novel Testosterone-regulated Apoptosis Inducer

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ABSTRACT

Androgens control prostate homeostasis and regulate androgen response genes. Here, we report the identification and characterization of U19, a novel testosterone-regulated apoptosis inducer with tumor suppressive activity. U19 is an evolutionarily conserved protein expressed in many human tissues, with the most abundant expression in the prostate, bone marrow, kidney, and lymph nodes. Overexpression of U19 in 12 surveyed cell lines induced apoptosis, and new protein synthesis is required for apoptosis induction. Expression of U19 in xenograft prostate tumors markedly induced apoptosis and inhibited tumor growth in vivo. Consistent with its tumor-suppressive role, U19 down-regulation was observed in all of the surveyed prostate cancer cell lines and in 19 of 23 clinical human prostate tumor specimens. Loss of heterozygosity analysis revealed U19 allelic loss in 19 of the 23 specimens. Furthermore, two of the specimens had homozygous U19 deletions, and one specimen had hypermethylated U19 promoter, indicating that U19 can be inactivated genetically or epigenetically. These observations suggest that U19 is growth inhibitory and tumor suppressive and that the disruption of androgen-dependent growth inhibition via U19 down-regulation is commonly associated with prostate cancer progression.

INTRODUCTION

Androgens play an important role in prostate cancer progression. It is generally thought that higher than usual levels of androgens is a risk factor for prostate cancer because androgens are required for prostate growth (1). Humans with androgen deficiency, such as eunuchs or individuals with inactive 5α-reductase, have underdeveloped prostate, and no prostate cancer case has been reported among these individuals (1). Androgen administration has been demonstrated to induce or accelerate prostate cancer in some animal models (2, 3). Furthermore, prostate cancers are, in general, androgen dependent initially and respond to androgen ablation therapy. The above-mentioned observations suggest that androgens are growth stimulatory in the prostate and that excessive androgen action is likely to be a causative factor in prostate carcinogenesis. However, this concept does not correlate with some observations. For example, as individuals age, the risk of prostate cancer increases dramatically, whereas androgen levels fall (4–6). The unusually high androgen levels may not exist in aging males and are thus unlikely to be a causative factor of prostate cancer.

Despite the different opinions discussed above, the importance of androgens in prostate cancer progression is well recognized. The elucidation of the androgen action pathway, a cascade of molecular and cellular events triggered by androgen manipulation leading to cell proliferation, apoptosis, and/or differentiation, would provide insights into the roles of androgens in prostate cancer progression.

The effects of androgens on the prostate are complex. The prostate will undergo extensive apoptosis and regression if androgens are ablated (7). Androgens stimulate proliferation in a regressed prostate, but not in a fully grown prostate. It was postulated by Bruchovsky et al. (8) that androgens induce not only mitogenic factors but also nullifiers that negate proliferation once the number of cells reaches the normal level. The homeostasis of the prostate should require balanced activities of the androgen-dependent mitogenic factors and nullifiers. Excessive activation of mitogenic factors and/or inactivation of nullifiers could conceivably lead to uncontrolled growth and, eventually, cancer. Because androgen action is mediated through androgen receptor, a ligand-dependent transcription factor (9), the mitogenic factors and nullifiers are likely to be encoded by androgen response genes. We have identified more than 20 androgen response genes in the rat ventral prostate using a gene expression screen (10). This report describes the characterization of one of the identified genes, U19 (10), as a novel testosterone-regulated apoptosis inducer with tumor-suppressive activity. Our studies provide new insights into the mechanisms of androgen action and the roles of androgens in prostate cancer progression.

MATERIALS AND METHODS

Cell Lines and Tissue Samples. The LNCap, PC3, DU145, and TSU cell lines were obtained from American Type Culture Collection. Dunning tumor cell lines G, AT1, AT2, AT3.1, AT6.1, and MatLyLu were provided by Allen Gao (University of Pittsburgh). The NIH3T3 cell line was provided by Ali Shah, and HeLa cells were provided by David Klump (Northwestern University). Human multiple tissue polyadenylated RNA Northern blot membranes were purchased from Clontech (catalogue numbers 7780-1 and 7784-1; Palo Alto, CA).

All archival patient specimens (Gleason score, 7–10) were from Department of Pathology, Northwestern Memorial Hospital. The specimens were cut into 5–7-μm sections and then stained with H&E on membrane-coated glass slides (catalogue number 11505134; Nuhbaum, McHenry, IL). About 5,000–10,000 cells from benign prostate tissue and distinct neoplastic foci were captured separately in the same stained sections using LCM1 (Leica LMD system; Nuhbaum). Genomic DNA was isolated and dissolved in a 50-μl final volume using the High Pure PCR Template Preparation Kit (Roche, Indianapolis, IN).

5′- and 3′-RACE and Low Stringency Hybridization. The 3′ region of mouse U19 cDNA was screened from a mouse cDNA library using low stringency hybridization with rat U19 cDNA as a probe. The 5′-RACE was performed for cloning the 5′ region of mouse U19 cDNA using primers 5′-GTCAACTCCACACAGTCACAG-3′, 5′-CGGTGACAAGACACATCACGC-3′, 5′-CTCAATGGTGCTGTTGCG-3′, and 5′-CACAATCTCATCTGTGTC-3′. Similarly, the 5′-RACE and 3′-RACE were performed for cloning the full-length of human U19 cDNA using primers 5′-TGATACTGGAGGATGTGGCGGC-3′, 5′-CACAATCTCATCTGTGCG-3′, 5′-CGGCTGGACATCTTATCTTC-3′, 5′-CAGTGGATTGTTGGCTGCTGAG-3′, and 5′-CTCAGCCAGCAATCATCTGTG-3′.

Vector Construction. The cDNAs of rat and human U19 were cloned into pEGFP C1, pEGFP N3, PM, and pIRE2-EGFP (Clontech) by PCR. Deletion mutagenesis of U19 was generated by PCR and cloned into above first three

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The abbreviations used are: LCM, laser capture microdissection; aa, amino acid(s); RACE, rapid amplification of cDNA ends; GFP, green fluorescent protein; 4-OHT, 4-hydroxytamoxifen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; LOH, loss of heterozygosity; ER, estrogen receptor; Z-VAD, Z-Val-Ala-DL-Asp-fluoromethylketone; ELL, eleven nineteen lysine-rich leukemia gene; TMR, tetramethyl rhodamine.
vectors. The plasmids containing ER domain were kindly provided by G. Evan (University of California San Francisco, San Francisco, CA). The ER ligand-binding domain was cut with BamHI/EcoRI and recloned into pEGFP-U19 to generate a tripartite fusion protein. All constructs were verified by sequencing and transfected using LipofectAMINE (Invitrogen) for LNCaP, DU145, TSU, and Dunning tumor cell lines: SuperFect reagent (Qiagen, Valencia, CA) for NIH3T3 and HeLa; or FuGENE 6 (Roche) for PC3. G418 (500 μg/ml) was used for stable selection of transfectants.

Cell Death Assays. In flow cytometry analysis, cells cultured under the indicated conditions were harvested for staining with the TACS Annexin V-Biotin Apoptosis Detection Kit (R&D Systems, Minneapolis, MN). Propidium iodide was used for nuclei staining, and Cy5 was conjugated to annexin V-biotin. Apoptosis of annexin V-positive cells was analyzed by flow cytometric analysis. Hoescht 33342 (Molecular Probes, Eugene, OR) was used for nuclear staining. DNA fragmentation assay was performed as described previously (11).

Androgen Protection Assay. LNCaP and PC3 cells were plated in a 6-well plate. Once the confluence reached 60–70%, GFP-tagged U19 was transiently transfected into LNCaP and PC3 cells using LipofectAMINE 2000 (Invitrogen). After the cells were incubated with mixture containing 1 μg of DNA, 5 μl of LipofectAMINE, and 1 ml of OPTI-MEM medium (Invitrogen) for 4–5 h, the mixture was replaced by 3 ml of RPMI 1640 with 10% fetal bovine serum. At the same time, mibolerone was added to the medium at a final concentration of 1 nm, and the same amount of ethanol vehicle was added to the control. After 24 and 48 h of transfection, the cells were observed via fluorescence microscopy. The detached cells and/or cells with fragmented nuclei were considered dead.

Tumor Growth. To determine their tumorigenicity, parental or stably transfected AT6.1 cells were injected s.c. (1 × 10^6 cells) into 4–6-week-old male nu/nu mice. To induce GFP-U19-ER activity in nude mice, tamoxifen (Sigma) and the androgen induction of U19 were administered i.p. (10 mg/kg body weight/day) for 2 weeks, starting on day 1 after transfection. Two sets of PCR reactions were performed using genomic DNA from patient 1 and exon 3 of the U19 gene. For exon 3 amplification, the sense and antisense primers are 5′-TATATCCTTG-AAAATCGTC-3′ and 5′-ACTTACACGATTTACTCGATAC-3′, respectively. For exon 3 amplification, the sense and antisense primers are 5′-CAAGATTCTAACAGCACCAC-3′ and 5′-TTTTGCTAGTAGTCAC-3′. The primers for β-actin amplification were 5′-ATGGTAGATTATCGCACC-3′ and 5′-CACCACCCTGCTTGCTTCT-3′.

Analysis of U19 Promoter Methylation. Microdissected tumors and matching normal prostate tissues were obtained from resected tissues. DNA was treated with sodium bisulfite following the protocol as described previously (15). Two pairs of primers were designed for amplifying the promoter region, which produced fragments of 335 bp (from −398 to −63 bp) and 211 bp (from −301 to −90 bp). Primers used for the first PCR were MT-U19-F2 (5′-TTTGGAGTTGGGGTTTGC-3′) and MT-U19-R4 (5′-AAACACTA-3′). Primers used for the second PCR were MT-U19-F4 (5′-TTTTATAGAAATGTTGGGG-3′) and MT-U19-R5 (5′-AAATCACCCAATCCAC-3′).

RESULTS

Sequence, Expression, and Androgen Regulation of U19. We first cloned the full-length cDNA of rat U19 and subsequently isolated the full-length cDNAs of mouse and human U19 using low stringency hybridization coupled with 5′- and 3′-RACE. As shown in Fig. 1A, mouse and rat U19 proteins are both 262 aa in length and share 91.6% identity. Human U19 has a 3-aa deletion and a 1-aa insertion in the COOH-terminal region relative to the rodent U19 proteins and shares 79.4% and 80.2% identity with rat and mouse U19, respectively. An interesting structural feature of U19 is its serine-rich sequence from aa 174 through 205 (Fig. 1). The NH2-terminal portion of U19 (residues 1–113) is more conserved than its COOH-terminal region. The NH2-terminal portion also shares significant homology (about 40% identity) with putative protein identified in Caenorhabditis elegans, Dro sophila melanogaster, and Arabidopsis thaliana. GenBank database search showed that human U19 gene is localized to chromosome 3q13.

Human U19 mRNA is expressed in virtually all surveyed tissues, with the most abundant expression in the prostate, kidney, bone marrow, and lymph nodes (Fig. 1B). In LNCaP human prostate cancer cells, androgens induce both U19 mRNA and protein (Figs. 1, C–E), and the androgen induction of U19 mRNA partially resisted inhibition of protein synthesis (Fig. 1C). These findings suggest that androgenic regulation of U19 in the prostate is evolutionarily conserved and that U19 is a primary androgen response gene. The androgen induction of U19 expression occurs very rapidly, within 8 h after androgen treatment (Fig. 1E).

U19 Is a Potent Apoptosis Inducer. To further characterize the function of U19, we generated U19 fusion proteins with GFP tagged at its NH2 or COOH terminus. The GFP-U19 or U19-GFP expression vectors were transiently transfected into human prostate cancer cell lines (LNCaP, PC3, DU145, and TSU), rat Dunning prostate tumor cell lines [G, AT1, AT2, AT3.1, AT6.1, and M]), and non-prostatic cell lines (NIH3T3 and HeLa). GFP-U19 or U19-GFP was localized to the nuclei of all of the transfected cells (data not shown). All cells transfected with U19-GFP or U19-GFP expression vector exhibited chromatin condensation and plasma membrane blebbing (data not shown), indicative of apoptosis.

To determine whether U19 alone, without GFP fusion, is capable of inducing apoptosis, we cloned U19 into a bicistronic expression vector that drives the expression of GFP and U19 as separate proteins in the same cell. As expected, the expression of untagged U19 induced efficient apoptosis in the transfected cells (data not shown). These observations are consistent with our inability to stably transfect pros-
tate cancer cells with constitutive untagged or FLAG-tagged U19 expression vector (data not shown).

To regulate U19 activity for further functional studies, we established a tripartite fusion protein GFP-U19-ER consisting of GFP at the NH2 terminus, U19 in the middle, and a modified estrogen receptor ligand-binding domain (ER) at the COOH terminus. The activity of transcription factor-ER fusion proteins can be regulated by tamoxifen or 4-OHT but not endogenous estrogens (21, 22). In stably transfected PC3 cells, Western blot analysis showed that the GFP-U19-ER fusion protein is expressed (Fig. 2e). The GFP-U19-ER was primarily localized to the cytoplasm in the absence of ligand 4-OHT and was translocated into nuclei in the presence of 4-OHT (Fig. 2a). The activation of GFP-U19-ER caused chromatin condensation and enhancement of Hoechst staining of the nuclei (Fig. 2a). The U19-induced apoptosis is sensitive to caspase inhibitor Z-VAD (Fig. 2, a and b), indicating the involvement of caspases in U19-induced apoptosis. Agarose gel electrophoresis revealed genomic DNA fragmentation in U19-induced cell death (Fig. 2d). The above-mentioned results demonstrate that U19 induces extensive cell death via caspase-dependent apoptosis.

The GFP-U19-ER inducible system appears to be leaky because a significant percentage (12.0%) of the cells in PC3 sublines stably expressing GFP-U19-ER were apoptotic in the absence of ligand (Fig. 2, a and b). In contrast, less than 1% apoptotic cells were detected by Hoechst staining in PC3 sublines stably expressing GFP-ER (Fig. 2b) or in parental PC3 cells (data not shown). This leaky U19 activity most likely reflects a low level of ligand-independent nuclear translocation of GFP-U19-ER (Fig. 2a). As expected, the leaky activity-induced apoptosis can be blocked by caspase inhibitor Z-VAD (Fig. 2, a and b). The induction of apoptosis by leaky U19 activity in transfected cells argues that U19 is a highly potent apoptosis inducer.

U19-induced apoptosis is sensitive to protein synthesis inhibition. In the absence of protein synthesis inhibitor cycloheximide, flow cytometric analysis by Annexin V staining detected 13.2% apoptotic cells in the absence of 4-OHT and 69.6% apoptotic cells in the presence of 4-OHT (Fig. 2c). In the presence of cycloheximide, the percentage of apoptotic cells was 19.1% in the absence of 4-OHT and 25.1% in the presence of 4-OHT, indicating that cycloheximide inhibited the apoptosis induced by 4-OHT. This observation suggests that U19-dependent transcription and new protein synthesis are required for the apoptosis induction.

**Androgen Inhibits U19-induced Apoptosis in LNCaP Cells.** Although U19 induced dramatic apoptosis in prostate cancer cells, its androgen-dependent expression does not cause apoptosis in the normal prostate. Also, the induction of U19 in androgen-sensitive human LNCaP cells by androgens does not induce cell death. This led us to hypothesize that androgens are protective against U19-induced apoptosis. We showed in Fig. 3 that mibolerone, a synthetic androgen, significantly inhibited U19-induced apoptosis in LNCaP cells. After 48 h of transfection, only 10.1% of the U19-transfected cells were dead in the presence of 1 nm mibolerone, whereas about 80.1% of the U19-transfected cell were dead in the absence of mibolerone. In contrast, mibolerone did not affect the apoptosis induced by U19 in androgen-insensitive PC3 cells, indicating that the androgen protection requires the androgen receptor. This observation provides an explanation for the fact that androgen induction of U19 in LNCaP cells does not induce cell death.

**Suppression of Xenograft Tumor Growth by U19 Expression.** The availability of prostate cancer cells stably expressing GFP-U19-ER provided an opportunity to test whether U19 can suppress tumor growth. The parental PC3 prostate cancer cells are aggressive and readily generate xenograft tumors when implanted s.c. (23). As expected, implantation of the parental PC3 cells or PC3 sublines expressing GFP-ER yielded aggressive xenograft tumors in all injected nude mice (data not shown). However, the PC3 sublines expressing GFP-U19-ER were unable to generate xenograft tumors after s.c. injection into nude mice, suggesting that the weak activity of GFP-U19-ER suppressed PC3 tumor growth in vivo in the absence of ligand. Thus, we examined the impact of U19 in the more aggressive AT6.1 rat Dunning prostate tumor cell line (12, 20). The AT6.1 sublines expressing GFP-U19-ER were able to generate
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Fig. 2. Apoptosis induction by U19. a, activation of the tripartite GFP-U19-ER fusion protein by 4-OHT in stably transfected PC3 cells. The top left panel shows the localization of GFP-U19-ER protein in the absence of 4-OHT, as visualized under green fluorescent microscope. The bottom left panel shows the localization of the fusion protein 24 h after treatment with 300 nM 4-OHT. The top middle panel shows Hoechst staining in the absence of 4-OHT. The bottom middle panel shows Hoechst staining 72 h after 4-OHT treatment. The top right panel shows the effect of caspase inhibitor Z-VAD (100 μM) in the absence of 4-OHT, and the bottom right panel shows the effect of caspase inhibitor Z-VAD (100 μM) in the presence of 4-OHT. Z-VAD was added 1 h before the addition of 4-OHT.

xenograft tumors in the absence of tamoxifen, but at a much slower rate relative to the parental or GFP-ER-transfected AT6.1 cells (Fig. 4a), suggesting that the AT6.1 xenograft tumors were also inhibited by leaky U19 activity. As expected, the growth of xenograft tumors derived from an AT6.1 subline expressing GFP-U19-ER was significantly inhibited (P < 0.005) by tamoxifen administration that would activate the U19 fusion protein (Fig. 4b). In contrast, tamoxifen administration had no detectable influence on the growth of xenograft tumors derived from the GFP-ER-transfected AT6.1 cells in parallel experiments (Fig. 4a). These observations showed that U19 markedly suppressed AT6.1 xenograft prostate tumor growth in vivo.

TUNEL assay was used to determine whether U19 induces apoptosis in vivo. In xenograft tumors of AT6.1 cells expressing GFP-U19-ER, 34.9% of the cells were TUNEL positive in the absence of ligand (Fig. 4c), suggesting that the leaky GFP-U19-ER activity also induced apoptosis in vivo. This observation is consistent with dramatically reduced growth of GFP-U19-ER xenograft tumors in the absence of tamoxifen. As expected, tamoxifen administration enhanced the TUNEL-positive cells to 61.0% in vivo (Fig. 4c). The control experiments showed no TUNEL staining in xenografts derived from AT6.1 cells expressing GFP-ER in either the absence or presence of tamoxifen (Fig. 4c; data not shown). These observations indicate that U19 induces apoptosis in prostate cancer cells in vivo.

Down-Regulation, LOH, Deletion, and Promoter Methylation of U19 in Human Prostate Cancer Specimens. If U19 is indeed playing an important tumor-suppressive role in prostate cancer, its expression should be frequently down-regulated in prostate cancer cells. Northern blot analysis showed that U19 expression in human prostate cancer cell lines LNCaP, PC3, DU145, and TSU is down-regulated relative to its expression in human benign prostatic hyperplasia tissues (Fig. 5a). Consistent with the suppressive role of U19 in prostate cancer progression, the level of U19 mRNA in LNCaP is greater than that in PC3, DU145, and TSU, which inversely correlates with the aggressiveness of these cell lines (Fig. 5a). U19 expression is also down-regulated in the Dunning rat prostate cancer cell lines (Fig. 5b). Down-regulation of U19 in all of the surveyed prostate cancer cell lines suggests that it is a key molecule in the growth control of prostate cancer.
To determine whether U19 protein is down-regulated in human prostate cancer specimens, we have generated an antihuman U19 antibody for immunohistochemistry studies. Typical U19 down-regulation is shown in Fig. 6e. Benign prostatic epithelial cells were strongly stained in the presence of anti-U19 antibody. In contrast, prostatic cancer cells exhibited weak or no staining by anti-U19 antibody. Significantly U19 down-regulation in advanced prostate cancer cells (Fig. 6d) was observed in 19 of 23 radical prostatectomy specimens with Gleason scores of 7–10 (Fig. 6c) obtained from patients naïve to androgen ablation.

To investigate whether U19 locus exhibits allelic loss, deletion, or mutation in neoplastic foci of clinical prostate cancer specimens, we performed LCM on samples from the same 23 patients. A very high rate of allelic loss was found for polymorphic markers encompassing the U19 locus on chromosome 3q13 (Fig. 6f, a and b). Of the 23 samples analyzed, 19 (82.6%) displayed LOH of the U19 locus (Fig. 6f). Furthermore, two of these 23 specimens had homozygous deletion of the U19 gene, one at exon 2 and the other at exon 3 (Fig. 6f).

Gene silencing that arises from methylation is an important epigenetic mechanism of tumor suppressor inactivation. Epigenetic inactivation of gene expression often involves complete transcription silencing by methylation of CpG islands in promoter regions. According to our computer analysis, the U19 promoter region from –302 to –110 bp relative to the transcription start site contains a CpG island with a CpG frequency more than 100 times higher than that of other U19 genomic regions. The bisulfite sequencing method was used to detect U19 promoter methylation using DNA from the 23 paired LCM samples. Due to technical difficulties, only 8 of 23 paired samples could be amplified by PCR. One of the specimens showed clear U19 promoter methylation in the tumor cells but not benign cells (Fig. 6g).

**DISCUSSION**

The present study describes the characterization of U19, an androgen response gene in the prostate. Our studies showed that U19 overexpression induces massive apoptosis and inhibits prostate tumor growth *in vivo*. We have also presented evidence for frequent U19 down-regulation and allelic loss in human prostate cancer specimens. Furthermore, we detected homozygous U19 deletion and promoter hypermethylation. These observations argue that part of the androgen action pathway is proapoptotic and/or growth suppressive and is frequently inactivated in prostate cancer progression.

U19 induces apoptosis and inhibits prostate tumor growth. Our transient transfection studies showed that U19 induced apoptosis in all of the surveyed prostatic and non-prostatic cell lines. This observation is consistent with our inability to establish stable prostate cancer cell clones with constitutive U19 expression vectors. However, we were able to establish prostate cancer cell lines with stable expression of the GFP-U19-ER fusion protein that allows the regulation of U19 activity by tamoxifen or 4-OHT. The GFP-U19-ER fusion protein has leaky U19 activity that induces apoptosis and can be further activated by 4-OHT to induce more dramatic apoptosis in prostate cancer cells in both culture dish and xenograft tumors. The fact that leaky U19 activity is sufficient to induce apoptosis argues that U19 is a highly potent apoptosis inducer. Consistent with its proapoptotic activity, U19 overexpression dramatically inhibited xenograft tumor growth of both PC3 and AT6.1 prostate cancer cells.

U19-induced apoptosis is a caspase-dependent process because of its sensitivity to the caspase inhibitor Z-VAD (Fig. 2, a and b). The mechanism by which U19 activates the caspase cascade remains to be elucidated. However, transcriptional activation of new genes appears
to be required for the apoptosis because U19 induction of apoptosis can be blocked by a protein synthesis inhibitor (Fig. 2c). Identification of U19 partners and/or downstream genes would provide insights into the mechanism of U19 action.

**Roles of U19 in Androgen Action.** It is difficult to predict whether endogenous U19 plays a role in apoptosis in the prostate, although its overexpression is highly apoptotic in cultured prostate cancer cells and in xenograft tumors. U19 is unlikely to be involved in the castration-induced apoptosis in the normal prostate because U19 expression is down-regulated by castration. Although U19 is expressed in the normal prostatic epithelial cells, these cells do not undergo extensive apoptosis in testis-intact animals. One possibility is that U19 may function differently in the normal prostate relative to prostate cancer. Alternatively, androgens can protect prostatic epithelial cells from U19-induced apoptosis. Kimura et al. (24) recently reported that androgens can inhibit apoptosis of androgen-sensitive LNCaP human prostate cancer cells via blockage of caspase activation in both intrinsic and extrinsic cell death pathways. As expected, U19-induced apoptosis, a caspase-dependent process (Fig. 2), is markedly inhibited by androgen in LNCaP cells (Fig. 3).

U19 is likely to play a growth-suppressive role in androgen action in the prostate because it is an apoptosis inducer that markedly suppresses xenograft tumor growth. Androgens are well known to regulate the growth of the prostate. However, the effect of androgens on prostate growth is complex. Androgens are growth-stimulating only in a regressed prostate, but not in a fully grown prostate, suggesting that androgens induce nullifiers that restrict growth once the prostate reaches the normal size (8). Because U19 is proapoptotic and/or growth suppressive, it may function as a nullifier that contributes to the androgen-dependent growth restriction in the prostate. Future studies such as targeted deletion of the U19 gene in mice will further define the roles of U19 in androgen action in the prostate.

**Roles of U19 in Prostate Cancer Progression.** Our studies suggest that U19 is a potential tumor suppressor in the prostate. Consistent with its potent apoptotic and tumor-suppressive activities, the expression of U19 appears to be incompatible with prostate cancer cells. No U19 mRNA expression was detected by Northern blot in the Dunning rat prostate cancer cell lines, and U19 expression in the human prostate cancer cell lines is much lower than that in the benign prostatic tissues. U19 down-regulation and allelic loss were observed in >80% human prostate cancer specimens, which is similar or higher than the inactivation of known tumor suppressors including RB (25–27), p53 (28–30), PTEN (31), and NKX3.1 (32). Our data suggest that U19 is a strong candidate tumor suppressor in the 3q13 region.

The effect of abnormal U19 inactivation by genetic or epigenetic mechanisms in prostate cancer cells is different from the effect of U19 down-regulation caused by falling levels of androgens. Androgens induce not only growth-inhibitory proteins such as U19 but also...
growth-stimulatory proteins. Down-regulation of growth-inhibitory genes along with down-regulation of growth-stimulatory genes due to low androgen levels is unlikely to predispose someone to prostate cancer. However, abnormal down-regulation of growth-inhibitory genes, without coordinated down-regulation of growth-stimulatory genes, could lead to uncontrolled growth in the prostate.

Our finding that U19 expression is frequently down-regulated in prostate cancer specimens and cell lines suggests that inactivation of androgen-dependent growth restriction pathway, via U19 down-regulation, is common in prostate cancer progression. Androgen receptor is present and functional in most human prostate tumors before androgen ablation therapy because the tumor cells express androgen-dependent prostate-specific antigen. Thus, the down-regulation of U19 is unlikely to be due to the lack of functional androgen receptor because most, if not all, radical prostatectomy tumor specimens were derived from patients positive for serum prostate-specific antigen and naïve to androgen ablation treatment. In the absence of U19 but in the presence of androgen receptor, androgens may be likely to cause excessive proliferation in the prostate and enhance cancer progression.

During the preparation of this manuscript, Simone et al. (33) reported that human EAF1, a homologue of U19, is a novel ELL-associated factor. Very recently, the same group reported human EAF2, which is identical to human U19 (34). EAF1 and U19/EAF2 share 58% identity and 74% aa conservation. As indicated in Fig. 1, U19 consists of a stretch of a serine-rich region spanning aa residues 174 through 205. However, no recognizable motif was identified in EAF1 and U19/EAF2. The NH₂-terminal region (aa 17–104) of U19/EAF2 can interact with ELL, and it remains to be determined whether ELL-EAF2/U19 interaction is critically important in apoptosis induction by U19. The COOH terminus (aa 177–260) of U19/EAF2 contains a transactivation domain (33, 34), suggesting that U19/EAF2 may function by influencing transcription. This concept is consistent with our data that new protein synthesis is required for U19 induction of apoptosis (Fig. 2c). More structural-functional studies will be required to characterize U19/EAF2.

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