LEF1 in Androgen-Independent Prostate Cancer: Regulation of Androgen Receptor Expression, Prostate Cancer Growth, and Invasion

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Abstract

A major obstacle in treating prostate cancer is the development of androgen-independent disease. In this study, we examined LEF1 expression in androgen-independent cancer as well as its regulation of androgen receptor (AR) expression, prostate cancer growth, and invasion in androgen-independent prostate cancer cells. Affymetrix microarray analysis of LNCaP and LNCaP-AI (androgen-independent variant LNCaP) cells revealed 100-fold increases in LEF1 expression in LNCaP-AI cells. We showed that LEF1 overexpression in LNCaP cells resulted in increased AR expression and consequently enhanced growth and invasion ability, whereas LEF1 knockdown in LNCaP-AI cells decreased AR expression and, subsequently, growth and invasion capacity. Chromatin immunoprecipitation, gel shift, and luciferase assays confirmed LEF1 occupancy and regulation of the AR promoter. Thus, we identified LEF1 as a potential marker for androgen-independent disease and as a key regulator of AR expression and prostate cancer growth and invasion. LEF1 is highly expressed in androgen-independent prostate cancer, potentially serving as a marker for androgen-independent disease.

Introduction

A variety of theories have been proposed regarding the mechanism by which prostate cancer cells progress to aggressive phenotypes, including increased invasive and metastatic potentials, and developing resistance to androgen ablation therapy (1–3). Of great importance, increasing evidence fortify that increased expression is also linked to cross-talk with growth factor receptor or other signaling pathways (16–24). LEF1, lymphoid enhancer-binding factor 1 in the Wnt signaling pathway, has been indicated to regulate AR expression (25). LEF1/TCF is the nuclear transducer on activated the Wnt pathway. Members of the family represent a group of proteins with DNA-binding activities that specifically recognize and bind to a contiguous six-base consensus sequence, 5'-a/t-a/t-c-a-a-g-3' within the 5' promoter regions of Wnt target genes (26). There are eight putative LEF1 binding sites described at the AR promoter region regulating its expression (25).

In addition to its function in a variety of developmental processes including stem cell renewal, embryonic development, and tissue differentiation, more recent evidence indicates that aberrant activity of the Wnt signaling pathway is involved in the tumorigenesis of several organ systems including androgen-independent prostate cancer (27–31). Androgen-independent prostate cancer cells are defined by their ability to survive testosterone deprivation by castration and to sustain AR activation through ligand-dependent and ligand-independent mechanisms (32–34). Several AR-positive androgen-independent prostate cancer cell lines have been established (12, 35, 36). In comparison with traditional AR-negative PC3 (or low AR) and DU145 cells, AR-positive androgen-independent cell lines are very useful in studying the growth and invasion characteristics of androgen-independent prostate cancer. Like other androgen-independent prostate cancer cell lines, LNCaP-AI cells can grow both in the presence and absence of androgen, whereas its parental LNCaP cells can only grow in the presence of androgen (15, 33, 37–40). This study used androgen-independent prostate cancer cells to determine the function and mechanism of LEF1 in the regulation of AR expression, prostate cancer growth, and invasion.

Materials and Methods

Cell culture, dual luciferase assays, cell proliferation, and in vitro Matrigel invasion assays. LNCaP cells were maintained in RPMI 1640 (Life Technologies) with 10% heat-inactivated bovine serum (fetal bovine serum). The androgen-independent LNCaP-AI cells were maintained in RPMI 1640 containing 1% charcoal-stripped, heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc.) and 5 μg/mL of insulin, as described previously (38). Dual luciferase assay was performed as previously described (38) with luciferase reporter pAR-Luc (with 1.7 kb AR promoter sequence) and pCMVLEF1 (OriGene). Cell proliferation by cell counting, flow cytometry, and Matrigel invasion assays were performed as described previously (41). For invasion assays, the percentage of invasion was expressed as the ratio of invading cells over cell number normalized on day 2 of the growth curve.

Immunofluorescence microscopy. The paraformaldehyde-fixed (4%) LNCaP and LNCaP-AI cells were permeabilized in methanol/acetone
(1:1) for 10 min at −20°C. The cells were incubated overnight at 4°C with anti-AR and anti-LEF1–specific antibodies diluted 1:500 in 5% bovine serum albumin buffer followed with anti-rabbit and anti-mouse IgG-Cy2–conjugated antibodies (Molecular Probes; 1:300 in 5% bovine serum albumin) incubation for 45 min at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (10 µg/mL; Molecular Probes) according to the manufacturer’s instructions.

**Chromatin immunoprecipitation assays.** Chromatin immunoprecipitation (ChIP) was performed as described (42) with the following modifications: cross-linking was initiated with 11% formaldehyde solution at room temperature for 10 min and then stopped by the addition of 0.125 mol/L of glycine. The cross-linked chromatin was sonicated with Sonifier 450 microtip (Branson Ultrasonic, Corp.) at power setting 2 for 10 × 25 s on ice, produced DNA fragments with an average size of 1 to 3 kb. For immunoprecipitation, 2 µg of anti-AR antibody, anti-LEF1 antibody, and IgG were mixed with 25 µg of the purified cross-linked chromatin and incubated overnight at 4°C. Immunocomplexes were washed with radioimmunoprecipitation assay buffer and TE buffer. After reversal and recovery of the immunoprecipitated chromatin DNA, the final DNA pellets were dissolved with 100 µL of water. Immunopurified DNA (4 µL) was used for a PCR with primers described in Table I.

**Gel shift assay.** The assay was performed as previously described (43). Nuclear proteins extracted from LNCaP or LNCaP-AI cells were reacted with 32P-labeled LEF1 binding sequence oligos corresponding to the promoter of the AR gene (25), in binding buffer containing 1 µg of poly(dIdC) for 30 min at room temperature. The reaction mixtures were then subjected to electrophoresis and the binding complexes were visualized by exposure on X-ray films. The specificities of the LEF-binding complexes were established using specific and nonspecific competitors (43), and LEF1 antibody.

**Western blot and immunohistochemistry analysis.** Whole cell or cell fraction extracts (42) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. Blots were incubated with primary antibodies (AR, LEF1, and β-actin; Cell Signaling Technology, Inc.) for 2 h at room temperature, washed with TBST, and incubated for 1.5 h with the horseradish peroxidase–conjugated secondary antibody (1:5,000; Amersham Biosciences). The protein bands were detected by an enhanced chemiluminescence kit (Amersham Biosciences). Immunohistochemistry for LEF1 and AR was performed using single-label immunohistochemistry on a NexES automated immunostainer as described previously (44).

**Results**

**High levels of LEF1 expression in androgen-independent disease.** In Affymetrix microarray with a U133A GeneChip (15), the ratio of LEF1 expression between LNCaP-AI (an androgen-independent variant of LNCaP; ref. 35) and LNCaP was 100-fold. To confirm this dramatic change of LEF1 expression and its cellular localization between LNCaP and LNCaP-AI cells, we performed reverse transcription-PCR (RT-PCR), Western blot analysis, and immunofluorescent microscopy. LEF1 showed sharply increased expression in LNCaP-AI at the mRNA level by RT-PCR (Fig. 1A, left, rows 1 and 2) and at the protein level by Western blot analysis (Fig. 1A, left, rows 3 and 4). Cell fractionation revealed that LEF1 is localized to the nucleus in LNCaP-AI cells (Fig. 1A, right, row 1) with nuclear colocalization of AR (Fig. 1A, right, row 2) and histone 4 (Fig. 1A, right, row 4), serving as controls for nuclear localization, and β-actin (Fig. 1A, right, row 3) as control for the cytoplasmic localization. Immunofluorescent microscopy confirmed the nuclear localization of LEF1 in the majority (98%) of LNCaP-AI cells (Fig. 1B). In contrast, there is minimal LEF1 expression in LNCaP cells. As a control, AR was localized to the nucleus in both LNCaP and LNCaP-AI cells (Fig. 1B, middle). We also examined the levels of LEF1 expression in several prostate cell lines, including benign immortalized RC165 and RC170 (45), PC3 (low or negative AR), and DU145 (negative for AR) cells and did not detect LEF1 expression by Western blot analysis (data not shown).

Because LEF1 is specifically increased in LNCaP-AI prostate cancer cells, we examined the expression of LEF1 in androgen-independent cancer using a tissue microarray consisting of 99 cases, including 24 benign, 56 androgen-dependent, and 19 androgen-independent cases of prostate cancer. Androgen-dependent specimens were derived from patients who were diagnosed with prostate cancer by transurethral resection of the prostate, having a high grade (Gleason score 8 or higher), and volume of disease. Androgen-independent samples were derived from patients who underwent transurethral resection of the prostate at least 6 months after surgical orchectomy. The immunostaining signal is scored as a combination of intensity (0 as negative, 1+ as weak, 2+ as moderate, and 3+ as strong expression) and percentage of positive cells (<10% as 1+ and ≥10% as 2+) as in any given case with the highest score as 5. Of 24 benign cases, LEF1 is not expressed in luminal cells in all cases and is only expressed in basal cells in certain cases (Fig. 1C, left). LEF1 is expressed in 9 of 56 (16%) androgen-dependent cases ranging from 20% to 60% positive cells for a given case. We observed a statistically significant ($P < 0.01$) difference of LEF1 expression between androgen-dependent and androgen-independent cases. LEF1 is expressed (Fig. 1C, right and Fig. 1D, left) in 13 of 19 (66%) androgen-independent cases. The expression of combined scores is statistically significantly

### Table 1. Primers used in this study

| RT-PCR | AR       | 5′-GCCAGGGGACATGTGTTCGCGCC-3′, 5′-CCTCTTGTAGTTCAGATCAC-3′ |
|        | Lef1     | 5′-TTCAAGGAGGGGCGAT-5′, 5′-TGATTACCGAAAATCTCAG-3′ |
|        | GAPDH    | 5′-GACATCGACGCGATCTTCT-3′, 5′-CAATACGGCCAAATCCGTGAC-3′ |
| Chip   | Site 1   | 5′-CTGAGTTGGGCTTGGTGGGC-3′, 5′-CCTTCTGATATCTCTCCTGTC-3′ |
|        | Site 2   | 5′-CAGAATGCGGCTTCTATG-3′, 5′-TGTCCTGATGACCTCAGC-3′ |
|        | Site 3   | 5′-GCCAAGAAATCCTCGGATGAC-3′, 5′-AAAGGATTGAAGTGCAAGTTG-3′ |
|        | Site 4/5  | 5′-ATCGATGTTGTCTGCTTGTTTT-3′, 5′-CTGTGGAGCTTCTGTCG-3′ |
|        | Site 6   | 5′-CTGGTTCAGGCTGTCGTCG-3′, 5′-AGGACATAATTTGTTCTGTTGCC-3′ |
|        | Site 7   | 5′-CAGGCTTGTTGTCAGG-3′, 5′-AAGGGTGAAGAATATAGTGGCAAGTG-3′ |
|        | Site 8   | 5′-CTGTCCTTTGTCCTGTTGTC-3′, 5′-AAGGGTTGAAAGTGAATAGTGGCAAGTG-3′ |
|        | PSA      | 5′-GGGAGGTCCACCTGCTGAG-3′, 5′-CCTGTCCTGCTGCTGCTG-3′ |
| Cyclin D1 |         | 5′-GCCAGGGGACATGTGTTCGCGCC-3′, 5′-CCTCTTGTAGTTCAGATCAC-3′ |
LEF1 is highly expressed in LNCaP-AI but not in LNCaP cells. In androgen-free medium, although androgen can stimulate its growth (Fig. 2B, left and right, respectively), validating the androgen-independent nature of LNCaP-AI cells. Flow cytometric analysis of LNCaP-AI cells with reduced levels of AR by short interfering RNA (siRNA) knockdown showed a 5.1% decrease of cells in the S phase of the cell cycle. Furthermore, we used Matrigel invasion assays to determine the difference of invasion ability between LNCaP and LNCaP-AI cells, both in androgen medium and in androgen-free medium. LNCaP and LNCaP-AI cells showed comparable minimal invasion in androgen-free medium (Fig. 2C and D, left). Although androgen increased the invasion ability of both LNCaP and LNCaP-AI cells, LNCaP-AI cells showed a greater increase in invasive ability (up to 4-fold) compared with LNCaP cells (1.5-fold) in the presence of 10 nmol/L of R1881 (Fig. 2D, left), suggesting that androgen and AR plays a key role for invasion in LNCaP-AI cells. We used small RNA interference technology to knockdown AR in LNCaP (Fig. 2A, middle) and LNCaP-AI cells (Fig. 2A, right) followed by invasion assays to confirm the AR-dependent nature of invasion. In the presence of 10 nmol/L of R1881, there was a 50% reduction in invasion by LNCaP cells and an even greater reduction (up to 3-fold) of invasive LNCaP-AI cells (Fig. 2D, right).

There was no statistical difference in invasiveness of both LNCaP and LNCaP-AI cells with (Fig. 2D, right) or without (Fig. 2D, left) AR knockdown in androgen-free medium. These results strongly indicate that increased invasiveness of LNCaP-AI cells is dependent on AR.

**Increased growth and invasion of LNCaP-AI is AR-dependent.** AR is expressed in both LNCaP and LNCaP-AI cells, with greater levels in LNCaP-AI cells (Fig. 2A, left). The increase in cell proliferation among LNCaP-AI cells is prominent in androgen-free medium, although androgen can stimulate its growth (Fig. 2B, left and right, respectively), validating the androgen-independent nature of LNCaP-AI cells. Flow cytometric analysis of LNCaP-AI cells with reduced levels of AR by short interfering RNA (siRNA) knockdown showed a 5.1% decrease of cells in the S phase of the cell cycle. Furthermore, we used Matrigel invasion assays to determine the difference of invasion ability between LNCaP and LNCaP-AI cells, both in androgen medium and in androgen-free medium. LNCaP and LNCaP-AI cells showed comparable minimal invasion in androgen-free medium (Fig. 2C and D, left). Although androgen increased the invasion ability of both LNCaP and LNCaP-AI cells, LNCaP-AI cells showed a greater increase in invasive ability (up to 4-fold) compared with LNCaP cells (1.5-fold) in the presence of 10 nmol/L of R1881 (Fig. 2D, left), suggesting that androgen and AR plays a key role for invasion in LNCaP-AI cells. We used small RNA interference technology to knockdown AR in LNCaP (Fig. 2A, middle) and LNCaP-AI cells (Fig. 2A, right) followed by invasion assays to confirm the AR-dependent nature of invasion. In the presence of 10 nmol/L of R1881, there was a 50% reduction in invasion by LNCaP cells and an even greater reduction (up to 3-fold) of invasive LNCaP-AI cells (Fig. 2D, right).

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**LEF1 promotes the growth and invasion of LNCaP-AI cells.** LEF1 is highly expressed in LNCaP-AI but not in LNCaP cells (Fig. 1A, left). To determine whether LEF1 is involved in the increased growth and invasion of LNCaP-AI cells, we performed cell proliferation and invasion assays of LNCaP with LEF1 overexpression and of LNCaP-AI cells with decreased LEF1 expression by siRNA knockdown. The levels of LEF1 expression in LNCaP cells transfected with pCMV:LEF1 were comparable with LEF1 in LNCaP-AI cells (data not shown). With LEF1 overexpression (Fig. 3A, top left), increases were observed in both LNCaP-LEF1 cell proliferation, up to 4.1% in S phase cell population by flow cytometry (Fig. 3B, left) and invasion, and up to 2.5-fold by Matrigel invasion assays (Fig. 3B, right). On the other hand, with reduced levels of LEF1 by siRNA (Fig. 3A, right), LNCaP-AI cell growth was reduced by 6% in S phase cell population in the androgen medium (Fig. 3C, left). Similarly, the invasion ability of LNCaP-AI cells was also reduced 3-fold in androgen-free media (Fig. 3C, right). To examine the specificity of the LEF1 knockdown and to guard against off-target effects, we repeated this experiment using two additional LEF1 siRNAs and obtained similar effects in cell growth and invasion assay (data not shown). Importantly, these distinct LEF1 siRNAs also substantially reduced AR expression in LNCaP-AI cells. Taken together, the results show that LEF1 is involved in the growth and invasion of LNCaP-AI cells.

**LEF1 regulates the expression of AR in LNCaP-AI cells.** AR is expressed at a greater level in LNCaP-AI than in LNCaP cells. In
parallel, LEF1 is expressed at high levels in LNCaP-AI and at minimal levels in LNCaP cells. We determined the relationship between the expression of LEF1 and AR in LNCaP and LNCaP-AI cells by siRNA knockdown of either AR or LEF1, coupled with Western blot analysis to detect LEF1 and AR expression, respectively. The LNCaP-AI cells with LEF1 knocked down by 10 nmol/L of siRNA exhibited a complete block in AR expression, shown by RT-PCR at mRNA levels (Fig. 4A, left), and by Western blot analysis at the protein level (Fig. 4A, middle). In contrast, knockdown of AR did not affect the levels of LEF1 expression in both LNCaP and LNCaP-AI cells due to decreased AR levels. AR was knocked down with siRNA at a final concentration of 10 nmol/L. In the presence of 10 nmol/L of R1881, LNCaP-AI cells exhibited a 2.5-fold reduction in invasiveness.

We performed ChIP and gel shift assays to examine the direct occupancy of LEF1 on the eight putative LEF1-binding sites of AR promoter in both LNCaP and LNCaP-AI cells. In LNCaP cells, we first confirmed the promoter occupancy of the three reported binding sites (25), sites 2, 3, and 4/5 (Fig. 4B, middle), confirming our ChIP assay data. In LNCaP-AI cells without androgen, LEF1 was readily recruited to sites 2, 3, and 4/5 of the AR promoter (Fig. 4B, bottom). After treatment of LNCaP-AI cells with androgen, LEF1 recruitment increased in both sites (2 and 4/5) as well as in intensity on the promoter of AR (Fig. 4B, bottom).

The specific binding of LEF1 on the AR promoter region was confirmed by gel shift assays using both wild-type and mutant probes. Data indicate that LEF1 binds to the site 2 binding region in LNCaP (Fig. 4C, lane 2) and LNCaP-AI cells (Fig. 4C, lane 3). The specificity of binding was confirmed as the complex does not form in the absence of probe (Fig. 4C, lane 1) and in the presence of mutant probes (5'-CCAGACTCTCGAGCGAAAATC-3'; Fig. 4C, lanes 4–6). Binding at sites 3 and 4/5 were also confirmed (data not shown), and there is no complex binding for site 6 (Fig. 4C, lanes 7–9).

To correlate the physical occupancy of LEF1 on the AR promoter with transcriptional activation of AR, we performed dual luciferase assays with a luciferase reporter containing a 6 kb AR promoter region in LNCaP cells. Transcriptional activity was increased up
to 2-fold with LEF1 overexpression (Fig. 4D). The results of the above studies strongly support the direct regulation of AR expression by LEF1.

Discussion

The AR expressing androgen-independent prostate cancer cells can grow in the absence of androgen, however, they grow faster in the presence of androgen (32). Our data show enhanced growth of LNCaP-AI cells in both androgen-free and androgen media, indicating the possible androgen-independent function of AR in this process. We also investigated the invasion of LNCaP and LNCaP-AI cells with a Matrigel invasion chamber. Both LNCaP and LNCaP-AI cells have higher invasive abilities in the presence of androgen, suggesting that androgen and AR are involved in invasion. In addition, LNCaP-AI cells showed >4-fold increases of invasion compared with LNCaP cells in androgen medium. AR knockdown by siRNA revealed reduced invasion of LNCaP-AI cells, indicating that the increased invasiveness of LNCaP-AI cells is androgen receptor–dependent, consistent with recent reports (12).

Interestingly, the enhanced invasion ability is also LEF1-dependent and our data showed that this is due to the regulation of AR expression by LEF1. Similar effects were observed with another set of androgen-dependent and androgen-independent MDA and MDA-HR cells. Wnt signaling has been shown to be involved in prostate cancer development and progression (27, 28, 31, 46) with cross-talk between the Wnt and androgen signaling pathways in prostate cancer (47, 48). β-Catenin, a key component of the Wnt signaling pathway, serves as a coactivator for LEF/TCF transcriptional activator proteins and a ligand-dependent coactivator of AR (49, 50). Wnt signals are transmitted to the nucleus through β-catenin and lead to active transcriptional regulation by LEF1 (51). There is very low expression of LEF1 in LNCaP cells but >100-fold increases of LEF1 expression in LNCaP-AI cells. On the protein level, LEF1 is localized to the nucleus in LNCaP-AI cells and binds to promoter regions of AR shown by ChIP and gel shift assays. Furthermore, when overexpressed, LEF1 increases levels of AR expression in LNCaP cells by Western blot analysis, and when knocked down by siRNA, LEF1 reduces the levels of AR expression in LNCaP-AI cells. Functionally, dual luciferase assays showed that LEF1 increased luciferase activity using a reporter expressing AR promoter. The results of this study provide a novel mechanism for the cross-talk between AR and Wnt pathways in androgen-independent prostate cancer cells. It is noteworthy to mention, however, that AR does not seem to be regulated by LEF1 at all times. For example, AR is expressed in prostate stromal cells (52),

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whereas LEF1 is not (Fig. 1C), suggesting the AR expression can be LEF1-independent. Also, during prostate development, AR is expressed in both epithelial and stromal cells; however, LEF1 is only expressed in a subset of epithelial cells. These data indicate spatial, temporal, and tissue-specific regulation of AR by LEF1.

The Affymetrix study with LNCaP and LNCaP-AI cells also revealed increased expression of other LEF1 target genes such as \(\text{MMP7}^7\). It is of great interest to determine the contribution of these LEF1 target genes in the growth and invasion of androgen-independent cancer cells. In addition, our data confirming LEF1 expression in both human androgen-independent prostate cancer and AR-positive, androgen-independent prostate cancer cell lines strongly indicate that LEF1 is associated with androgen-independent prostate cancer and may potentially serve as a marker for androgen-independent disease. Given the importance of LEF1 in androgen-independent LNCaP-AI cell growth and invasion, it will be important to test and compare the growth of LNCaP-AI, LNCaP-LEF1, and LNCaP-AI-LEF1 siRNA cells in an animal model in the future. This could lead to a new mouse model for metastatic and androgen-independent prostate cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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