Amyloid Precursor Protein Is a Primary Androgen Target Gene That Promotes Prostate Cancer Growth

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Abstract

Androgen receptor (AR) is a critical transcription factor that regulates various target genes and contributes to the pathophysiology of prostate cancer hormone dependently. Here, we identify amyloid precursor protein (APP) as a primary androgen target through chromatin immunoprecipitation (ChIP) combined with genome tiling array analysis (ChIP-chip). ChIP-treated DNA were obtained from prostate cancer LNCaP cells with R1881 or vehicle treatment using AR or acetylated histone H3 antibodies. Ligand-dependent AR binding was further enriched by PCR subtraction. Using chromosome 21/22 arrays, we identified APP as one of the androgen-regulated genes with adjacent functional AR binding sites. APP expression is androgen-inducible in LNCaP cells and APP immunoreactivity was correlated with poor prognosis in patients with prostate cancer. Gain-of-function and loss-of-function studies revealed that APP promotes the tumor growth of prostate cancer. The present study reveals a novel APP-mediated pathway responsible for the androgen-dependent growth of prostate cancer. Our findings will indicate that APP could be a potential molecular target for the diagnosis and treatment of prostate cancer. [Cancer Res 2009;69(1):137–42]

Introduction

Prostate cancer is originally an androgen-responsive tumor, the growth of which could be initially controlled by hormone therapy consisting of a combination of antiandrogenic agents. Most prostate cancer cases, however, will eventually fail to later respond to the endocrine treatment (1). Androgen receptor (AR) has been revealed to play a critical role in both androgen-dependent and androgen-refractory prostate tumors (2), although the pathogenesis of the disease remains unclear. Thus, exploring essential androgen-dependent miotgens for prostate cancer would be the first step for the development of alternative therapeutic options.

Amyloid precursor protein (APP) is a type I transmembrane protein that includes several human isoforms due to alternative splicing. One of the processed APP products, β-amyloid, is considered to be central in the development of Alzheimer’s disease (3). The affected regions of Alzheimer’s disease patients’ brains are characterized by amyloid plaques, in which the neurotoxic β-amyloid peptides are major components. APP is also ubiquitously expressed in nonneuronal tissues, and might also be involved in the growth of various cell types in both physiologic and abnormal states. The studies using fibroblasts revealed that APP is secreted to the cell culture medium and have an autocrine function in growth regulation (4). The secreted APP is also observed in the culture medium of tumor cells such as rat pheochromocytoma PC12 cells (5) and human lung squamous cell carcinoma cells (6).

In the process of screening androgen-dependent AR-binding sites (ARBS) in human prostate cancer LNCaP cells, we performed human genome tiling array analysis using DNA obtained from chromatin immunoprecipitation (ChIP), or ChIP-chip, and identified that APP was one of the genes that include bona fide ARBSs in their transcription-regulatory regions. We show that APP is a primary androgen-responsive gene that promotes the growth of prostate cancer cells, and knockdown of APP in mice represses tumor growth. Moreover, APP expression is a potential tumor marker that predicts the outcome of prostate cancer. The present study implicates APP as a primary AR effecter that contributes to prostate cancer progression.

Materials and Methods

ChIP, LNCaP cells, after 72 h of hormone depletion, were treated with R1881 (10 nmol/L) or vehicle (0.1% ethanol) for 24 h. ChIP assay followed by quantitative PCR was performed as described (7). Fold enrichment of ChIP samples was determined by normalizing to input control. Antibodies used were AR (H-280) from Santa Cruz Biotechnology, acetylated histone H3 (AcH3) from Upstate Biotechnology, and RNA polymerase II (Pol II, 8WG16) from Covance. AcH3 ChIP DNA fragments were amplified by two cycles of in vitro transcription as described (8, 9).

PCR amplification combined with ChIP DNA subtraction. AR recruitment of ChIP DNA was further enriched by PCR subtraction based on representational difference analysis. Subtractive hybridization was performed between adapter-ligated target amplicon (AR ChIP DNA from R1881-treated LNCaP cells) and a 10-fold excess amount of driver ampiclon (adapter-removed AR ChIP DNA from vehicle-treated LNCaP cells) using GeneFisher PCR Subtraction System (TaKaRa Bioc). PCR amplification was performed with primers specific for the adaptors. The above kinetic enrichment was repeated twice.

ChIP-chip. Subtracted AR ChIP DNAs and in vitro translation–amplified AcH3 ChIP DNAs were fragmented and end-labeled with biotin. Two
micrograms of ChIP and nonenriched control samples were hybridized on separate Affymetrix GeneChip chromosome 21/22 tiling arrays. Array data were quantile-normalized and analyzed as described (8). Probe pairs were mapped on chromosomes 21/22 based on the human genome version NCBI Build 33. AR and AcH3-enriched regions were determined at a P value cutoff of 1e-4.

Sequence analysis. Sequences for human RefSeq transcripts (hg 15) were retrieved from the UCSC Genome Browser (10). Enrichment analysis of androgen response elements (ARE) was performed based on a position-weighted matrix method, using position-weighted matrices retrieved from TRANSFAC. The threshold score of the ARE motif was 1e-4 against the background.

Reverse transcription-PCR. Total RNA extraction, first-strand cDNA synthesis, and quantitative PCR were performed as described (7, 8). Fold induction of mRNA by androgen was determined by comparing the mRNA levels of the ligand-treated samples with those of the vehicle-treated control.

Western blot analysis. Western blot analysis was performed as described (8). Anti-APP antibody (Cell Signaling) was used for the detection of APP protein.

Luciferase assay. Luciferase reporter constructs containing APP 3′ (ARBS #11), APP 3′ with mutation (ARBS #11 with mutated ARE), and APP intron 1 (ARBS #12) regions were generated using pGL3 promoter plasmid (Promega). LNCaP cells were plated in 24-well culture plates at a density of 10,000 cells/well in phenol red–free medium containing 5% charcoal-stripped serum. Luciferase activity was evaluated using LNCaP cells transfected with reporter genes in the presence of vehicle (open bars) or R1881 (10 nmol/L, closed bars) for 24 h.

Figure 1. Hormone-dependent AR and AcH3 binding sites in the vicinity of the APP gene in LNCaP cells. A, PCR subtraction for ChIP-chip. ChIP was performed by anti-AR in cells treated with vehicle or R1881 (10 nmol/L) for 24 h. Adaptor-ligated ChIP DNA of R1881-treated cells were hybridized with 10 times the amount of ChIP DNA of vehicle-treated cells. Nonhybridized DNA were amplified with primers specific for the adaptors. DNA fragments were biotin-labeled and applied to the chromosome 21/22 array. B, genomic mapping of ARBSs and AcH3 binding sites (AcH3BS) close to APP on chromosome 21 in LNCaP cells (NCBI 33). C, hormone-dependent recruitment of AR, RNA polymerase II (Pol II), AcH3, and SRC1 on ARBSs and AcH3BS close to APP. LNCaP cells were treated with vehicle (open columns) or R1881 (10 nmol/L, closed columns) for 24 h. D, promoter activity of ARBSs close to APP. Luciferase reporter genes were constructed by inserting genomic fragments including ARBSs #11 and #12 into the pGL3-enhancer vector. Both inserted fragments included consensus ARE sequences as determined by TRANSFAC (threshold, >75% matrix conservation). APP 3′ mut Luc was generated from APP 3′ Luc by mutating two bases positioned at ±2 bp from the three-base spacer. Luciferase activity was evaluated using LNCaP cells transfected with reporter genes in the presence of vehicle (open bars) or R1881 (10 nmol/L, closed bars) for 24 h.

Patients and tissues. Tumor specimens were obtained from 104 patients who agreed with informed consent and underwent radical prostatectomy between 1987 and 2001 at the Tokyo University Hospital. The mean patient age was 66.8 years (range, 52–78), the mean preoperative prostate-specific antigen was 16.9 ng/mL (2.2–136), and the mean follow-up period was 82 months (10–192). Thirty-three tumors were stage B, 59 were stage C, and 12 were stage D1. Twenty-three tumors were Gleason 2 to 6, 41 were Gleason 7, and 40 were Gleason 8 to 10 (as evaluated by two pathologists; T. Suzuki and H. Sasano). Thirty-five patients were treated with surgery alone, whereas 69 patients received adjuvant antiandrogen therapy.

Immunohistochemistry. Immunohistochemistry for APP was performed as described, based on the streptavidin-biotin amplification method using formalin-fixed and paraffin-embedded tissue specimens (11). The antigen-antibody complex was visualized by 3,3′-diaminobenzidine.
Statistical analyses. Cancer-specific survival rates were evaluated by the Kaplan-Meier method, and statistical significance between APP-negative and APP-positive groups was determined using a log-rank test.

Proliferation assay. LNCaP clones stably expressing APP (APP #3 and #9) and control pTRE-hyg2 vector (vector #1 and #2) were selected by hygromycin B (150 μg/mL). For experiments with recombinant human APP peptide (R&D Systems), cells were seeded in 96-well plates at a density of 3,000 to 5,000 cells/well 1 day prior, and incubated with the soluble APP peptide or vehicle for 72 h. Cell proliferation was examined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide (a tetrazole) assay kit (Nacalai Tesque).

Small interfering RNA. Small interfering RNA (siRNA) were synthesized by RNAI, Co., Ltd. (Japan) and transfected into LNCaP cells using Lipofectamine 2000 (Invitrogen). siRNA sense sequences were 5′-GUCCUGACAAUUGAAUUC-3′ for control (siCTRL), and 5′-GAUCAUAGGGACCAAAAACC-3′ and 5′-GUCCUGACAAUUGAAUUC-3′ for APP (siAPP-A and siAPP-B, respectively).

Results

Ligand-dependent ARBSs and histone acetylation sites in androgen-sensitive prostate cancer cells. To obtain AR ChIP DNA for arrays, we performed subtractive hybridization between R1881-treated and vehicle-treated LNCaP samples (Fig. 1A). AR binding in the androgen-responsive prostate-specific antigen promoter (∼250/∼39 bp from the transcriptional start site) was
APP expression at both mRNA and protein levels was androgen-inducible (Fig. 2A and B). Notably, the molecular weight of the major APP isoform in LNCaP cells was close to that of APP-770 expressed in 293T cells (Fig. 2B). Immunohistochemistry showed that the androgen-dependent expression of APP protein was predominantly in the cytoplasm of LNCaP cells (Fig. 2C).

**APP immunostaining is positively associated with the prognosis of patients with prostate cancer.** To investigate whether APP expression plays a role in the progression of prostate cancer, we evaluated the immunostaining of APP in prostate cancer specimens. The quality of the anti-APP antibody was validated with a brain specimen from a patient with Alzheimer’s disease (Supplementary Fig. S1). In normal prostate, no APP immunoreactivity was basically detected (Fig. 3A). In prostate cancer, intense and diffuse APP staining was mainly observed in the cytoplasm (Fig. 3B and C). Fifty-eight of 104 prostate tumors were positively immunostained by anti-APP (Fig. 3D). The rate of cancer-specific survival for patients with APP-positive tumors was significantly lower than those with APP-negative tumors. At the end of the study period, patients with APP-positive tumors showed 50% survival compared with nearly 100% of those with APP-negative tumors. The pathologic study suggests that APP is a poor prognostic factor for prostate cancer.

**APP contributes to androgen-dependent proliferation of prostate cancer cells.** To further assess the role of APP in prostate cancer, we performed gain-of-function and loss-of-function studies for APP. APP-overexpressing LNCaP cells exhibited a significantly higher growth compared with control cells expressing empty vector (Fig. 4A and B). We also confirmed that the recombinant soluble APP, which could be endogenously produced as the secretory NH2-terminal ectodomain of APP by cleavage at the a-secretase site, significantly promoted the growth of LNCaP cells (Fig. 4A).

**Figure 4.** APP overexpression promotes in vivo proliferation of androgen-dependent prostate cancer cells. A, generation of LNCaP clones stably expressing APP or control vector. Western blotting was performed using anti-APP (top) and anti-β-actin (bottom). B, APP overexpression accelerates the growth of cultured LNCaP cells. Cell proliferation was analyzed by MTT assay. Points, means of four wells; bars, SE. *, P < 0.05 for mean absorbance of APP #3 and #9 vs. mean value of vector #1 and #2. C, recombinant soluble APP (sAPP) accelerates the growth of cultured LNCaP cells. Columns, means of three to four wells; bars, SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs. vehicle-treated cells at indicated times.

increased by 6.7-fold and 40-fold after the first and the second PCR, respectively, compared with pretreatment DNA. We also obtained ACh3 ChIP DNAs by in vitro translation amplification to investigate ligand-dependent histone acetylation sites.

**APP is a primary androgen-regulated gene in the vicinity of functional AR and ACh3 binding sites.** Screening ARBSs by ChIP-chip in chromosomes 21/22, we identified novel ARBSs in the 3' downstream region and intron 1 of APP (ARBSs #11 and #12, respectively; Fig. 1B). In intron 1 of APP, close to its transcription start site, we found a ligand-inducible ACh3 site (ACh3BS #28; Fig. 1B). We also confirmed a functional ARBS in the 5' region of TMPRSS2 (12), which was also shown in ChIP-chip by Wang and colleagues (13). Ligand-dependent AR-binding was validated for ARBSs #11/#12 and ACh3BS #28 (Fig. 1C). ARBS #11 and ACh3BS #28 exhibited ligand-induced Pol II binding, and ARBS #11 also exhibited SRC1 binding (Fig. 1C). Luciferase reporter assay revealed that AREs in ARBSs #11/#12 showed ligand-dependent transcriptional activity (Fig. 1D).

**Figure 5.** APP knockdown impairs protein expression and reduces LNCaP cell growth. A, APP-specific siRNAs impair APP protein expression in cultured LNCaP cells. Cells were transfected with 200 nmol/L of control siRNA (siCTRL) or two different siRNAs targeting APP (siAPP-A and siAPP-B) for 48 h. B, APP repression by specific siRNAs (200 nmol/L each) inhibits androgen-dependent growth of cultured LNCaP cells. *, P < 0.05 vs. siCTRL. Columns, means of four wells; bars, SE.
For APP knockdown, we used two APP-specific siRNAs (siAPP-A and siAPP-B; Fig. 5A). Both siRNAs could substantially reduce endogenous APP expression in LNCaP cells. In proliferation assays, R1881-dependent growth of cultured LNCaP cells was significantly repressed by the APP-specific siRNAs, whereas basal growth of cells was apparently not affected by the treatment (Fig. 5B).

Moreover, athymic male mice bearing LNCaP cell–derived tumors were treated with siAPP-B or siCTRL. Tumor formation was prominent in mice treated with siCTRL, although it was substantially reduced in mice with siAPP-B (Fig. 6A and B). APP protein expression was almost abolished in tumors treated with siAPP-B (Fig. 6C). Taken together, APP is a novel androgen-regulated gene that could promote the growth of primary prostate cancer.

**Discussion**

The present study shows that APP is an androgen-regulated gene with adjacent functional AREs. APP expression is androgen-inducible in LNCaP cells and the intensity of APP immunostaining correlates well with the poor prognosis of patients with prostate cancer. APP overexpression and the soluble peptide accelerate the growth of tumor cells, and APP knockdown represses the proliferation of LNCaP cells in culture and nude mice. Our findings indicate that APP plays a critical role in the androgen-dependent growth of prostate cancer as a primary androgen target, which is a novel mediator of androgen signaling.

APP has been shown to function as a growth factor or a docking molecule in membrane proximal signaling events in nonneural epithelial cells and fibroblasts. The NH2-terminal domain of APP structurally resembles some growth factors, as it is cysteine-rich and contains a heparin-binding site (14). The protease-inhibitory domain, or Kunitz protease inhibitor domain, has been shown to require a mitogenic action of APP in fibroblasts (4, 5). The Kunitz protease inhibitor domain is contained in the nonneuronal isoforms including APP-770, APP-751, and APP-733, but not the neural isoform APP-695. The cytoplasmic tail of APP is also shown to bind to cytoplasmic proteins such as FE65 and Shc (15, 16). APP modulates cell motility and cell adhesion by interacting with the actin cytoskeleton, integrins, and extracellular matrix (17, 18). In monocytic lineage cells, APP is a critical component of integrin-mediated increase in cyclooxygenase-2 (COX-2), a key enzyme in the conversion of arachidonic acid to prostaglandins, suggesting the role of APP in proinflammatory activation (19). Interestingly, inflammation has been considered as a poor prognostic factor for prostate cancer, and COX-2 is indeed one of the therapeutic targets of prostate cancer. Thus, APP could also play a role in proinflammatory activation by interacting with COX-2 in prostate cancer.

APP also seems to function as a local mediator of growth in several malignant tumors such as lung (6), colon (20), parathyroid (21), pancreas (22), oral (23), and thyroid cancers (24). Nevertheless, the transcriptional regulation of APP has not been well investigated. For instance, thyroid hormone receptor–mediated repression of APP transcription has been previously reported in cultured neuroblastoma-derived cells (25), although the biological significance of the effect remains to be studied. The present findings will provide a new insight into the pathology of prostate cancer because APP has been shown as a potential key mediator of androgen-dependent growth mechanism.

Our findings will also have an effect on the clinical treatment of prostate cancer. The biological relevance of APP could be extended to the use of this factor as a potential molecular target for diagnosis and alternative therapeutics of prostate cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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