Conversion of Prostate Cancer from Hormone Independence to Dependency Due to AMACR Inhibition: Involvement of Increased AR Expression and Decreased IGF1 Expression

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Abstract. Androgen-independent prostate cancer eventually develops metastasis, and radical treatment may not be possible for patients at this stage. In this study, we examined the gene-expression profiles of two prostate cancer cell lines, LNCaP (androgen-dependent) and C4-2 (androgen-independent), using cDNA-microarray hybridization. We focused on the expression of α-methylacyl-CoA racemase (AMACR), whose expression is much higher in C4-2 than in LNCaP, and investigated its biological role in acquisition of androgen-independent cancer growth. Immunohistochemistry and Western blot analysis of subcellular fractions revealed that AMACR expression was much stronger in C4-2 than in LNCaP. Inhibition of AMACR expression using AMACR-siRNA induced an increase in the expression of androgen receptor (AR) and B-cell translocation gene 1, along with a decrease in the expression of genes associated with cancer progression, including insulin-like growth factor I and platelet-derived growth factor alpha, in C4-2 with compared to non-treated C4-2. BrdU analysis and MTT assay demonstrated that AMACR inhibition induced a significant decrease of cell viability in C4-2 when cultured in androgen-depleted serum, becoming consistent with that of LNCaP, suggesting that AMACR inhibition may induce an increase in the expression of AR and characteristic conversion of prostate cancer cells from hormone independency to hormone dependency. We suggest that AMACR inhibition may be a new strategy for treatment of patients with hormone-refractory prostate cancer.

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Carcinoma of the prostate now constitutes a major and escalating international health problem, and is the most common solid tumor malignancy in men in Western countries (1). There are several curative treatment options such as surgery or irradiation when patients are in the initial stage of disease. The fact that over 70% of prostate carcinomas rely on androgen stimulation for growth sets the basis for androgen ablation therapy, which is initially effective but invariably results in treatment resistance after a period of time (2). After initial regression, most prostate cancer becomes androgen-independent and progress further, with eventual fatal outcome.

Despite intensive studies over many years, the molecular events underlying the progression of prostate cancer from hormone-sensitive to a hormone-refractory state remain unknown. Because survival of patients after tumor progression is poor and alternative therapies are ineffective, identification of the molecular mechanisms associated with disease progression and failure of endocrine therapy is imperative. A large number of different molecular mechanisms might be responsible for the transition to androgen-independence. Recent loss-of-function studies have revealed that androgen receptor (AR) still plays a key role in hormone-refractory progression of prostate cancer (3, 4). An adaptation of AR signaling in order to function under low or absent androgen levels may occur (5). However, the biological behavior of prostate cancer cannot be accurately predicted through estimation of increased or diminished expression of any single gene or a small number of genes. Hence, analysis of the expression profiles of a large number of genes is an essential step toward understanding in detail the mechanisms of androgen independence in prostate cancer. We therefore conducted the present study to examine alterations in the gene expression patterns of 17,086 human genes in C4-2, an androgen-independent cell line, in comparison with LNCaP, an androgen-dependent cell line.

Among the genes showing increased expression in C4-2 compared with LNCaP, we focused on α-methylacyl-CoA
r Racemase (AMACR), whose expression is about 5 times higher in C4-2 than in LNCaP. AMACR is a peroxisomal and mitochondrial enzyme capable of racemizing the α-carbon of various α-methylacyl-CoA derivatives (6). Overexpression of AMACR has been identified in prostate adenocarcinoma and is recognized as a new immunohistochemical marker (7). A previous study has indicated that decreased levels of AMACR expression in prostate cancer cells lead to decreased proliferation and that this effect appears to be completely independent of androgen action (8), although details of the function and expression of AMACR remain unclear. In the present study, morphological analysis using confocal immunofluorescence microscopy and Western blot analysis revealed that AMACR was expressed mainly in organelles in both LNCaP and C4-2, while C4-2 showed much higher expression than LNCaP.

We next investigated the influence of AMACR expression on the expression pattern of other genes. We transfected chemically synthesized AMACR siRNA into C4-2 cells, and investigated changes in the pattern of gene expression using cDNA array analysis. We found increased expression of AR and B-cell translocation gene 1 (BTG1), along with decreased expression of genes associated with cancer cell proliferation, including insulin-like growth factor 1 (IGF1) and platelet-derived growth factor alpha (PDGFA), in C4-2 after AMACR inhibition, as compared with non-treated C4-2.

Our investigation of cell growth and microarray analysis has revealed for the first time that increased expression of AR and BTG1, and decreased expression of IGF1 and PDGFA due to AMACR inhibition may induce characteristic conversion of prostate cancer cells from hormone independency to hormone dependency. We suggest that AMACR could be a potential target for treatment of hormone-refractory prostate cancer.

Materials and Methods

Cell culture. The human prostate cancer cell lines LNCaP and C4-2 were obtained from Dr. L.W. Chung (University of Virginia, Charlottesville, VA, USA). They were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

RNA isolation and quality measurement. Total RNA was isolated from each sample using Trizol reagent (Invitrogen) in accordance with the manufacturer’s instructions. The eluted RNA was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The quality of the RNA was verified with an Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA).

Microarray analysis and data acquisition. A 17k cDNA microarray containing a set of 17,086 sequence-verified human cDNA clones (Invitrogen) was provided by Genomic Tree, Inc. (Daejon, South Korea). The synthesis of target cDNA and hybridization were performed in accordance with the manufacturer’s protocol. Microarray scanning was performed using a GenePix 4000B (Molecular Devices Corporation Sunnyvale, CA, USA) and analyzed using GenePix Pro6.0 (Molecular Devices). The average fluorescence intensity for each spot was calculated and local background was subtracted. All data normalization and selection of genes whose expression changed several fold were performed using Gene Spring 7.3 (Agilent Technologies).

Confocal immunofluorescence microscopy. Immunofluorescence examination of AMACR was carried out in LNCaP and C4-2. Cells (5x10⁶) were seeded in 8-well chamber slides with a collagen coat (BD Biosciences, San Jose, CA, USA) in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The primary antibodies used were anti-AMACR rabbit monoclonal antibody (Abcam, Cambridge, UK) and anti-AMACR rabbit polyclonal antibody (Chemicon, Temecula, CA, USA), both at 1:500 dilution. The secondary antibody was Alexa Fluor 488 anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) at 1/400 dilution. Nuclei were stained with PI solution (Dojindo, Kumamoto, Japan). Cells were washed five times in PBS, mounted on glass slides and visualized on an Olymusp Fluoview FV500 confocal laser scanning microscope.

Subcellular fractionation and immunoblotting. Purified proteins from four subcellular fractions of LNCaP and C4-2 – organelle, cytosolic, cytoskeletal and nuclear fractions – were obtained using a ProteoExtract Subcellular Proteome Extraction Kit (Merck, Darmstadt, Germany) in accordance with the manufacturer’s instructions. Five micrograms of protein from each subcellular fraction were loaded and separated using 15% SDS-polyacrylamide gel (Bio Craft, Tokyo, Japan) electrophoresis, and then electroblotted onto Immobilon-P Polyvinylidene Difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Immunoblotting was performed with the primary antibodies: anti-AMACR rabbit monoclonal antibody (Abcam) and anti-AMACR rabbit polyclonal antibody (Chemicon). Immunoblots were exposed to the secondary antibody, anti-rabbit IgG HRP-linked whole antibody, and visualized using ECL Plus chemiluminescence detection reagents (GE Healthcare, Uppsala, Sweden).

siRNA transfection in C4-2 (microarray analysis). Chemically synthesized AMACR siRNA and negative control siRNA (B-bridge International, Mountain View, CA, USA) were diluted in 500 μl of serum-free RPMI-1640 in one well of a 6-well tissue culture plate and mixed gently. Four microliters of Lipofectamine RNAiMAX (Invitrogen) was added to each well containing 30 pmol siRNA and incubated for 20 min at room temperature. The cells (at 70-90% confluence) were detached using trypsin-EDTA, resuspended in a volume of 3 ml into each well of a 6-well plate for 2-day transfection with siRNA and the Lipofectamine RNAiMAX mixture. After 48 h of incubation at 37°C in a humidified atmosphere containing 5% CO₂, the cells were harvested for microarray experiments to determine the knockdown level of the exogenous mRNA by the siRNA. For targeting and detection of the endogenous gene level, cells were transfected with the same concentration of negative control siRNA.

Cell growth assay. LNCaP and C4-2, and C4-2 treated with AMACR siRNA and negative control siRNA (B-bridge International) were seeded in each well of 96-well plates and maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, 100 units/ml...
penicillin and 100 μg/ml streptomycin or RPMI-1640 medium supplemented with 10% charcoal/dextran-treated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Transfections were carried out with a mixture of siRNA and Lipofectamine RNAiMAX transfection reagent (Invitrogen). The final concentrations of siRNA and Lipofectamine RNAiMAX used in each transfection were 10 nM and 0.12 μl, respectively. Cells were cultured for days 1, 2 and 3 at 37°C in a humidified atmosphere containing 5% CO₂. Each time course screening assay was performed using triplicate cultures for three independent experiments.

BrdU assay: BrdU assays were performed on days 1, 2 and 3 after LNCaP and C4-2, and C4-2 treated with AMACR siRNA and negative control siRNA seeding. For determination of DNA synthesis, the Cell Proliferation ELISA BrdU assay (Roche Molecular Biochemicals, Mannheim, Germany) was used in accordance with the manufacturer’s protocol. Briefly, cells cultured in 96-microwell plates were pulse-labeled for 2 h with 10 μM BrdU. The cells were then fixed and the DNA was denatured for 30 min. After 1.5 h of incubation with a peroxidase-coupled anti-BrdU-antibody, the cells were washed three times with PBS. Thereafter,
peroxidase substrate (tetramethylbenzidine) was added for 30 min and measurements were performed on a Model 680 Microplate Reader (Bio-Rad). Absorbance was measured at a test wavelength of 405 nm and a reference wavelength of 490 nm. The cells were treated with RPMI-1640 medium supplemented with 10% FBS or 10% charcoal/dextran-treated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

**MTT assay:** Cell viability of LNCaP, C4-2, C4-2 treated with AMACR siRNA, and C4-2 treated with negative control siRNA was assessed by MTT assay performing at three time points (on day 1, 2 and 3) after the seeding. Ten microliters of MTT reagent were added to each well of the plates and incubated for 4 h at 37˚C. After the reaction, 100 μl of isopropanol with 0.04 N HCl were added to each well. The plates were then gently shaken for 30 min at room temperature. Each reaction was transferred into a 96-well plate and absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 630 nm with a Model 680 Microplate Reader (Bio-Rad). Cells were treated with RPMI-1640 medium supplemented with 10% FBS or 10% charcoal/dextran-treated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

**Statistical analysis.** Unpaired Student’s t-test was applied to values obtained by in vitro MTT assay and BrdU analysis. Differences at p<0.05 were considered to be statistically significant.

**Results**

cDNA microarray analysis of the hormone-refractory prostate cancer cell line C4-2 and the hormone-sensitive prostate cancer cell line LNCaP. Analysis using a cDNA microarray containing a set of 17,086 sequence-verified human cDNA clones was performed on the hormone-refractory prostate cancer cell line C4-2 and the hormone-
sensitive prostate cancer cell line LNCaP. Among the genes related to prostate cancer, we found those whose expression levels were more than three-fold higher or less than one-third in C4-2 as compared with LNCaP. The results are presented as expression values in Table I. Among these genes, we focused on AMACR, whose expression was more than five times higher in C4-2 than in LNCaP, and investigated its biochemical / biological role in the acquisition of androgen-independent growth of C4-2, as AMACR is known to be overexpressed in prostate adenocarcinoma and is recognized as a new immunohistochemical marker (7).

Localization of AMACR expression. We first examined the localization of AMACR expression in LNCaP and C4-2 using confocal immunofluorescence microscopy. AMACR was expressed mainly in organelles, but not in the nucleus or cytoskeleton, in both cell lines. There was no significant difference in the pattern of AMACR expression between LNCaP and C4-2, while the level of expression was much higher in C4-2 than in LNCaP (Figure 1). In order to confirm the above results, we performed Western blot analysis on fractions of cytosol, organelles, nucleus, and cytoskeleton separated from each cell line using a ProteoExtract Subcellular Proteome Extraction Kit. The results clearly corroborated the morphological data obtained by confocal immunofluorescence microscopy. AMACR expression was evident mainly in organelles in both LNCaP and C4-2 cells, but with much higher expression levels in C4-2 than in LNCaP (Figure 2).

Changes in pattern of gene expression due to AMACR inhibition by siRNA-AMACR transfection in C4-2 cells: increased expression of androgen receptor and decreased expression of IGF-1. We next investigated the influence of AMACR expression on the expression pattern of other genes. We transfected chemically synthesized AMACR siRNA into C4-2 cells, and investigated the changes in the pattern of gene expression using cDNA array analysis. We found increased expression of AR and BTG1, along with decreased expression of genes associated with cancer cell proliferation, including IGF1 and PDGFA, in C4-2 after AMACR inhibition as compared to non-treated C4-2 cells. These results are presented as expression values in Table II.

Acquisition of androgen-dependent cell growth due to AMACR inhibition in C4-2. We then focused on the increased expression of AR due to AMACR inhibition, since AR is highly relevant to hormone-dependent prostate cancer cell growth, under the hypothesis that increased expression of AR due to AMACR inhibition may induce characteristic conversion of cancer cells from hormone independency to hormone dependency. We first examined the influence of AMACR inhibition on cell viability as well as cell growth using BrdU analysis and MTT assay. BrdU assay demonstrated that the viability of LNCaP cells significantly decreased when they were cultured with androgen-depleted serum as compared with medium containing the usual 10% level of FBS, whereas C4-2 cells grew normally in androgen-depleted serum; there were no significant differences in cell growth rate in comparison with culture in medium containing 10% FBS (Figure 3 a). In contrast, however, C4-2 cells treated with AMACR siRNA showed significantly decreased cell viability when they were cultured with androgen-depleted serum as compared with medium containing 10% FBS (Figure 3 a). The MTT assay clearly corroborated the above results: C4-2 cells treated with AMACR siRNA showed significantly decreased cell viability in the absence of androgen relative to that in medium containing 10% FBS, and untreated cells growing normally in culture medium with androgen-depleted serum (Figure 3 b). AMACR siRNA negative control transfection did not influence
the viability of either C4-2 or LNCaP cells in the BrdU assay or the MTT assay, the results being almost the same as those for cells without treatment. These results indicated that increased expression of AR due to AMACR inhibition may induce characteristic conversion of prostate cancer cells from a hormone-independent to hormone-dependent state, suggesting a promising avenue of treatment for hormone-independent prostate cancer.

**Discussion**

The molecular events underlying the progression of prostate cancer from a hormone-sensitive to a hormone-refractory state have remained unknown despite intensive studies over many years. Because survival of patients after tumor progression is poor and alternative therapies are ineffective, identification of the molecular mechanisms associated with disease progression and failure of endocrine therapy is an important goal. In order to investigate the molecular mechanism of androgen-independent growth of prostate cancer cells, we performed a microarray-based gene expression analysis of 17,086 transcripts in two biologically distinct human prostate carcinoma cell lines, LNCaP and C4-2. Among the cancer-related genes whose expression levels were more than three-fold higher or less than one-third in C4-2 as compared to LNCaP, we focused on AMACR and investigated its biochemical / biological role in the acquisition of androgen-independent growth of C4-2. Schmitz *et al.* first purified...
AMACR from human liver as a key factor involved in the β-oxidation of branched-chain fatty acids in 1995 (9). AMACR is a peroxisomal and mitochondrial enzyme capable of racemizing the α-carbon of various α-methylacyl-CoA derivatives (6). Specifically, AMACR is responsible for converting the α-methyl group of C27-bile acyl-CoAs and pristanoyl-CoA from (R) to their (S) stereoisomers, which are the only stereoisomers that can be degraded via β-oxidation in peroxisomes and mitochondria. Previous immunohistochemistry studies of AMACR in different stages/grades of prostate cancer have yielded different conclusions regarding this issue. Some studies have indicated that AMACR staining was absent in 55% of prostate adenocarcinoma cases after hormone therapy (10), and one set of studies from three different groups confirmed that the intensity of AMACR staining remains unchanged when prostate cancer progresses to a high grades or stage (11-13). On the other hand, other studies have identified a decrease of AMACR expression in hormone-refractory metastasis, compared with that in localized prostate adenocarcinoma (14, 15). Overexpression of AMACR has now been identified in prostate adenocarcinoma and is recognized as a new immunohistochemical marker (7). However, the reliability of AMACR as a diagnostic marker in post-hormonal therapy cases is controversial.

What is the function of AMACR? Previous studies have suggested multiple pathways through which metabolic enzymes might be involved in the growth regulation of cancer cells, e.g., maintaining essential energy equilibrium, preserving reduction/oxidation balance, and generating/degrading critical signaling molecules (16). Increased glycolysis and fatty acid synthetase have been described in various cancer types including prostate cancer (16). Among new therapeutic targets for treatment of prostate cancer, the fatty acid synthetase inhibitors cerulenin and C75 have been shown to have potent activity as selective inhibitors of tumor cell growth (17). β-Oxidation of branched-chain fatty acids such as pristanic acid might provide a critical energy source in the course of prostate tumorogenesis, and inhibition of this activity might slow tumor growth. An in vitro experiment has demonstrated that a decrease in the level of AMACR protein results in decreased proliferation of prostate adenocarcinoma cells, and that the function and expression of AMACR are independent of AR-mediated signaling (8). However, the growth inhibition of prostate cancer can be accelerated by androgen ablation to achieve an additional growth-inhibitory effect (8).

Although various studies have focused on AMACR recently, its role in hormone-refractory prostate cancer is still unclear. In the present study, we first examined the localization of AMACR expression in the cancer cell lines LNCaP and C4-2 using confocal immunofluorescence microscopy. AMACR was expressed mainly in organelles, and not in the nucleus or cytoskeleton, in both cell lines. There was no significant difference in the pattern of AMACR expression between LNCaP and C4-2, although the level of expression was much higher in the androgen-independent prostate C4-2 cancer cell than in the androgen-dependent prostate cancer cell, LNCaP. This suggests that AMACR may be involved in the mechanism of androgen-independent cell growth in prostate cancer cells. We then investigated the influence of AMACR expression on the expression patterns of other genes. We transfected chemically synthesized AMACR siRNA into C4-2 cells, and investigated the changes of gene expression pattern using cDNA array analysis. We found an increase in the expression of AR and BTG1, along with a decrease in the expression of genes associated with cancer cell proliferation, including IGF1 and PDGFA, in C4-2 after AMACR inhibition as compared with non-treated C4-2. The AR, which is a ligand-dependent transcription factor and member of the classI subgroup of the nuclear receptor superfamily, plays a key role in prostate carcinogenesis and progression. In response to androgen, cytoplasmic AR rapidly translocates to the nucleus and interacts with sequence-specific androgen response elements in the transcriptional regulatory regions of target genes (18, 19). In addition to this transcriptional genomic action, androgens and other steroid hormones like progesterone and estrogen can exert rapid nongenomic effects that are not mediated through nuclear receptors, but rather initiated at the plasma membrane, presumably through surface receptors (20, 21). Conversely, AR is known to be highly relevant to hormone refractory change. BTG1 may play a coordinated role in a general transduction pathway induced in response to DNA damage (22). BTG1 expression is maximal in the G0/G1 phases of the cell cycle and is down-regulated when cells progress throughout G1 (23). It affects proliferation by phosphorylating a putative p34cdc2 kinase site on BTG1, Ser159, thus modulating CCR4 expression, and then induces the formation of hCAF-1/BTG1, which is of great consequence in the signaling events of cell division that lead to changes in cellular proliferation associated with cell-cell contact (24). Flutamide treatment might up-regulate CDKN1A and BTG1 expression in prostate cancer cells (25). However, the role of this gene in prostate cancer cell proliferation and cell contact is unclear. IGF1 and PDGF have been receiving attention as genes related to the tumorogenesis of prostate cancer. Among the various suggested mechanisms by which AR may be reactivated in a low-androgen environment (26), signaling by growth factors, especially IGF1, is reportedly of significance (27-30). High IGF1 serum levels are correlated with an increased risk of prostate cancer (27, 28), whereas IGF1 enhances AR transactivation under very low or absent androgen levels (31, 32) and promotes prostate cancer proliferation (29). PDGF is synthesized by both osteoblasts and osteoclasts and is integral to regulation of the perpetual bone remodeling process (33).
PDGRFs have been implicated in the metastasis of prostate cancer to bone, and PDGFR overexpression has been reported in the majority of prostate cancer bone metastases as well as in primary prostate cancer (34). Although further studies will be necessary, our present results of cell growth and microarray analyses indicate that increased expression of AR and BTG1, along with decreased expression of IGF1 and PDGF due to AMACR inhibition, may suppress cell viability, and contribute to the characteristic conversion of prostate cancer cells from hormone independency to hormone dependency.

As there are currently no effective drug treatments for hormone-refractory prostate cancer, which ultimately metastasize and have a fatal outcome, we suggest that AMACR inhibition, which may induce characteristic conversion from a hormone-independent to dependent state, could be a promising new strategy for treatment of patients with hormone-refractory prostate cancer. Oral administration of AMACR inhibitors may be a treatment option with potentially good compliance, since the major clinical manifestation of congenital racemase deficiency is adult-onset sensory motor neuropathy caused by slow, prolonged accumulation of branched-chain fatty acids, although AMACR is present in many organs (35, 36). Moreover, our ongoing in vivo experiments involving local transfection of siRNA for AMACR into tumors have revealed characteristic conversion from a hormone-independent to a hormone-dependent state, eventually leading to significant tumor regression, thus suggesting the feasibility of targeted gene therapy for patients with hormone-refractory prostate cancer.

References


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