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13. ABSTRACT (Maximum 200 Words)

The present study examined the cellular and molecular effects of selenium in the androgen-responsive LNCaP cells. Physiological concentrations of selenium produced a dose- and time-dependent inhibition of growth with this cell line. An arrest at G_0/G_1 phase was observed at 24 h. A marked decrease in the transcript and protein level of androgen receptor (AR) and prostate-specific antigen was detected as early as 6 h, suggesting that the interference of androgen signaling by selenium is not a consequence of growth arrest. Additional experiments showed that selenium was capable of suppressing the DNA-binding and the *trans*-activating activity of AR. Gene expression profiling was carried out in order to identify other AR-targets responsive to selenium modulation. The analysis showed that selenium was able to countermand the expression of a subset of 12 additional AR-regulated genes. These genes all contain ARE motifs in their promoters. As a follow-up to the above investigation, we also found that selenium favorably altered the expression of 12 putative oncogenes, tumor-suppressor genes, or genes implicated in the transformation to androgen-independency. By extracting relevant information from the microarray data, we have thus uncovered a number of potentially exciting clues regarding the action of selenium in prostate cancer prevention.

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Summary:

A major goal of this project is to identify suitable biomarkers of selenium chemoprevention in human prostate cell models. During this second year of funding period, we investigated the cellular and molecular changes mediated by selenium in the LNCaP human prostate cancer cell line. Please see the attached article (Dong *et al.*, Cancer Res., 64, 19-22, 2004) and the manuscript (submitted to Cancer Res.) in Appendix for detailed description of the specific aspects of the research. In the original Statement of Work, we proposed to look at the LNCaP prostate cancer cell line in the first year and the PC-3 cell line in the second year. The reason that we switched the sequence of the work is that PC-3 cells are relatively easy to handle. We would like to work out the experimental conditions in PC-3 before we proceed to the LNCaP cell line. We also proposed to perform Affymetrix GeneChip analysis in normal prostate cells in order to identify gene expression changes induced by selenium. Based on our experience with the PC-3 cell line, we found that the GeneChip analysis would generate voluminous amount of data. The challenge is how to extract out critical information. Therefore, we developed a bioinformatics-based approach to further analyze our GeneChip data, as detailed in the following section. We would like to postpone the study in normal prostate cells until the bioinformatics-based approach is validated.

Detailed Description of the Promoter-Based Microarray Data Mining Approach:

In collaboration with Dr. Haitao Zhang, a bioinformatist at our Institute, we developed a novel bioinformatics-based approach to further analyze the Affymetrix GeneChip data generated in the PC-3 cells. The ultimate goal is to characterize key transcription factors that might mediate selenium-induced gene expression changes. The underlying premise of this approach is based on two fundamental principles: ♦ *Genes that are coordinately regulated should share common regulatory elements in their promoter regions.* ♦ *By identifying such common regulatory elements for a cluster of early selenium-responsive genes, we should be able to deduce the transcription factors associated with these elements. These transcription factors are potential proximal targets of selenium.*

To group genes based on their expression profiles across multiple time points, we used the Self Organizing Map (SOM) algorithm to do the analysis on all the genes that are significantly modulated by MSA. This mode of analysis produced 16 distinct clusters **(Fig. 1)**. We decided to select cluster 14 to pursue the bioinformatics interrogation as an exemplary case study. The characteristics of this cluster and the reasons for its selection are delineated below.

- ❖ There are 372 genes in the cluster; the sizable number should be sufficient to validate the feasibility and reliability of our bioinformatics approach.
- ❖ The genes in this cluster are all significantly downregulated (based on statistical computation) by MSA only at the 3-hr time point. The entire cluster returns to

control level of expression at the subsequent time points of analysis (*i.e.*, 6, 12, 24, 36 and 48 hr). This pattern is consistent with a direct mechanism of selenium control of the activities of transcription factors.

- The fact that these 372 genes are downregulated upon exposure to selenium implies that they are expressed constitutively. In the array data, the change in the expression of a gene that is constitutively expressed in the control samples is inherently more reliable compared to the change observed with a gene that is not normally expressed in the control samples.

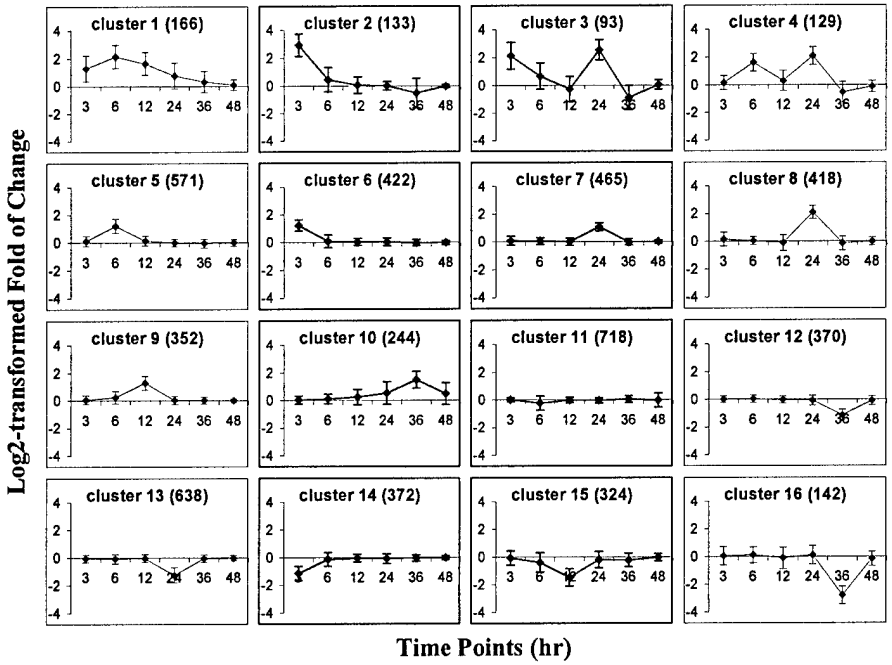

Fig. 1. Average gene expression profiles for each SOM cluster. The Log2-transformed fold of change for each gene in a cluster was averaged and plotted against duration of treatment. Data are presented as means ± SD. Numbers in parentheses represent the number of genes included in each cluster.

We were able to retrieve and analyze the promoter sequences of 287 genes (out of 372) with the custom Perl programs. The accession number of each transcript was used to map the transcript to a Unigene cluster and to obtain the Locus ID of the corresponding gene. The Locus ID was then used to query the NCBI LocusLink database in order to retrieve the unique reference sequence (RefSeq) of the gene. The latter was then matched against the human genome assembly at the University of California at Santa Cruz using the GoldenPath Genome Browser to obtain its mapping information. Based on this information, 1 kb of promoter sequence was retrieved for each gene. The transcription factor-binding motifs presented in each promoter were then profiled using the Match program and the TRANSFAC 6.0 transcription factor database. To control for background noise, each of the promoter sequence was scrambled to generate a random sequence of the same base composition. The scrambled sequence was then profiled for transcription factor-binding motifs. The process was repeated 10 times, and the average occurrence frequency for each binding motif was calculated and assigned as background.

The binding motifs with significantly higher frequencies (≥ 2 standard deviations) than the background noises were tabulated. The collective binding-motif profiles for all the promoters were then analyzed to search for common transcription factor-binding elements, and the corresponding transcription factors were classified as potential mediators of selenium action.

Table 1 shows a partial list of the common transcription factor-binding motifs identified in the promoters of the 287 genes analyzed. A substantial proportion of them (113/287 or 39%) contain the gut-enriched krüppel-like factor (GKLF)-consensus element in their promoter regions. The binding motifs for Sp1, Pax-4, Lyf-1, SRY, MZF1, C/EBP, and Elk-1 are also present at high frequencies. Since Sp1- and Lyf-1-consensus elements are highly present in TATA-less promoters, they might not be specifically involved in MSA-mediated transcriptional control but rather be important for regulating the constitutive expression of these genes. We are in the process of analyzing the promoters of two other clusters of early responsive genes, which were upregulated by MSA at the 3-hr time point. Clearly, our study demonstrates a novel approach of mining the large dataset generated by microarray analysis, and provides a paradigm of how to apply bioinformatics tools to investigate the molecular mechanism of not only cancer chemopreventive agents but also chemotherapeutic agents.

Table 1. Common Transcription Factor-Binding Motifs

Factor	Matrix	Frequency	Gene list
GKLF	M00286	113	click
Sp1	M00196	90	click
Pax-4	M00380	90	click
Lyf-1	M00141	78	click
SRY	M00148	61	click
MZF1	M00084	47	click
C/EBP	M00159	40	click
Elk-1	M00007	40	click

In order to confirm that GKLF is potentially a mediator of selenium action, we performed EMSA to assess the effect of MSA on the DNA-binding activity of GKLF. Nuclear extracts were prepared from PC-3 cells incubated in the absence or presence of 10 μM MSA for 3, 6, or 15 hr. A GKLF consensus element, 5'-ATGCAGGAGAAAGAA GGGCGTAGTATCTACTAG-3', was used as a probe in the EMSA. As shown in **Fig. 2**, MSA treatment resulted in an induction of GKLF DNA-binding activity. The specificity of the interaction and the presence of GKLF in the DNA-protein complex were confirmed by the competition experiment using an excess of unlabeled GKLF element as well as the supershift assay using an antibody against GKLF, respectively **(Fig. 2)**.

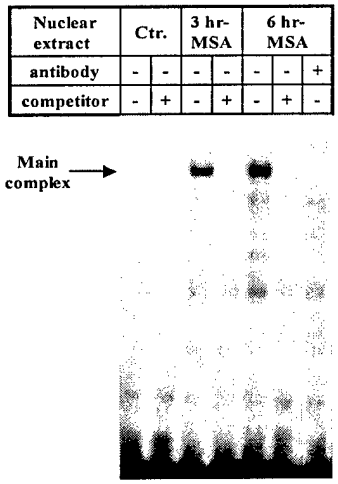

Fig. 2

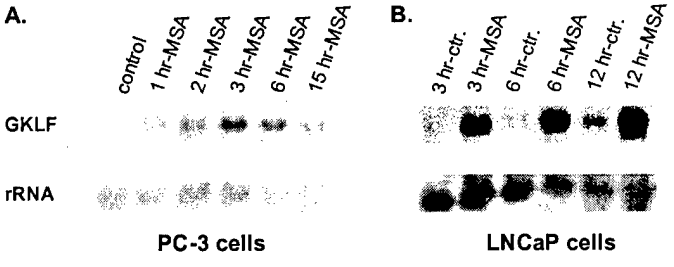

Fig. 3

Our microarray data showed that MSA upregulates the level of GKLF transcript signal in PC-3 cells. We therefore conducted Northern blot analysis to corroborate this observation. As shown in **Fig. 3A**, the increase

of GKLF mRNA level occurs as early as 1 hr post-MSA treatment. GKLF expression was also examined in LNCaP cells. The changes were very similar to that seen in PC-3 cells (**Fig. 3B**).

Key Findings:

- Selenium-induced growth inhibition in LNCaP cells is achieved mainly by G_0/G_1 cell cycle arrest coupled to an induction of apoptosis.
- Selenium significantly downregulates the expression of prostate-specific antigen (PSA) transcript and protein, and the decreases in androgen receptor (AR) transcript and protein follow a similar dose and time response pattern upon exposure to selenium. The reduction of AR and PSA expression occurs well before any significant change in cell number.
- Selenium inhibits the DNA-binding and the *trans*-activating activity of AR.
- Microarray analysis shows that selenium is able to countermand the expression of a subset of 12 additional AR-regulated genes. These genes contain the androgen responsive element motifs in their promoters. The information suggests that selenium might be able to directly modulating the primary targets of AR signaling.
- The array data also indicates that selenium is capable of favorably altering the expression of 12 putative prostate oncogenes, tumor-suppressor genes, or genes implicated in the transformation to androgen-independency.
- A bioinformatics-based array data mining approach was developed.
- GKLF was identified as a potential proximal target of selenium.

Reportable Outcomes:

- ***Publication and Manuscript:***

  Dong, Y., Lee, S.O., Zhang, H., Marshall, J., Gao, A., and Ip, C. (2004) Prostate specific antigen (PSA) expression is down-regulated by selenium through disruption of androgen receptor signaling. *Cancer Res., 64, 19-22.*

  Dong, Y., Zhang, H., Marshall, J., Nowak, N., Gao, A.C., and Ip, C. (2004) Selenium countermands the expression of androgen-regulated genes and other targets implicated in prostate carcinogenesis. *Cancer Res., submitted.*

- ***Abstracts and Presentations:***

  Dong, Y., Zhang, H., Nowak, N.J., Hawthorn, L., and Ip, C. (2003) A Bioinformatics Approach to Delineate Selenium-mediated Transcription Control of Gene Expression Changes. *Proceedings of the American Association for Cancer Research, 44 (2nd Edition): 774*

  Dong, Y., Zhang, H., Nowak, N.J., Hawthorn, L., and Ip, C. (2003) A Bioinformatics Approach to Identify the Molecular Mechanism of Selenium-mediated Gene Expression Changes. Poster presented at the Edward A. Smuckler Memorial Pathobiology of Cancer Workshop, Keystone, Colorado.

  Dong, Y., Zhang, H., and Ip, C. (2003) Selenium and genomics: a pas de deux to cancer control. Poster presented at the 2nd Annual International Conference on Frontiers in Cancer Prevention Research, Phoenix, AZ.

  Dong, Y., Zhang, H., Nowak, N.J., Marshall, J.R., and Ip, C. (2003) Selenium Affects an Array of Androgen-Regulated Genes and Other Targets Implicated in Prostate Carcinogenesis: Evidence from cDNA Microarray Analysis. Poster presented at the Prostate Cancer Foundation 10th Annual Scientific Retreat, New York, NY.

- ***Funding received:***

  DOD New Investigator Award (PI) 02/04 - 1/07

  - Title: GKLF as a Novel Target in Selenium Chemoprevention of Prostate Cancer
  - Award per year: $75,000

- ***Employment applied for:***

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[CANCER RESEARCH 64, 19–22, January 1, 2004]

Advances in Brief

Prostate Specific Antigen Expression Is Down-Regulated by Selenium through Disruption of Androgen Receptor Signaling

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Abstract

A previous controlled intervention trial showed that selenium supplementation was effective in reducing the incidence of prostate cancer. Physiological concentrations of selenium have also been reported to inhibit the growth of human prostate cancer cells *in vitro*. The present study describes the observation that selenium was able to significantly down-regulate the expression of prostate-specific antigen (PSA) transcript and protein within hours in the androgen-responsive LNCaP cells. Decreases in androgen receptor (AR) transcript and protein followed a similar dose and time response pattern upon exposure to selenium. The reduction of AR and PSA expression by selenium occurred well before any significant change in cell number. With the use of a luciferase reporter construct linked to either the PSA promoter or the androgen responsive element, it was found that selenium inhibited the *trans*-activating activity of AR in cells transfected with the wild-type AR expression vector. Selenium also suppressed the binding of AR to the androgen responsive element site, as evidenced by electrophoretic mobility shift assay of the AR-androgen responsive element complex. In view of the fact that PSA is a well-accepted prognostic indicator of prostate cancer, an important implication of this study is that a selenium intervention strategy aimed at toning down the amplitude of androgen signaling could be helpful in controlling morbidity of this disease.

Introduction

Prostate cancer is the second most common cancer as well as the second most common cause of cancer death in men in the United States. Every year, there are ~190,000 new cases and 30,000 deaths from prostate cancer (1). Age is a major risk factor; the incidence is 1 in 53 for men in their 50s but 1 in 7 for men from 60 to 80 years of age. A chemopreventive modality that can suppress or delay the clinical symptoms of prostate cancer would be well suited for preserving the quality of life in high-risk elderly men. In a previous randomized, placebo-controlled cancer prevention trial in which prostate cancer was evaluated as a secondary end point (974 of the 1312 subjects in the cohort were men), supplementation with a nutritional dose of selenium was found to reduce prostate cancer incidence by 50% (2, 3). Recent studies by Dong *et al.* (4) showed that selenium inhibited human prostate cancer cell growth, blocked cell cycle progression at multiple transition points, and induced programmed cell death. Prostate specific antigen (PSA) is a gene known to be under the control of the androgen receptor (AR) and is a well-accepted marker for the diagnosis and prognosis of prostate cancer. In view of the clinical observation of the effectiveness of selenium in prostate cancer prevention, it is reasonable to believe that selenium might be able to reduce the expression of PSA. If confirmed, this attribute obviously has the advantage of forecasting the responsiveness to selenium intervention. In this report, we describe a series of experiments that were designed to test the hypothesis that selenium is capable of down-regulating PSA through a mechanism of attenuating the functional intensity of the AR signal transduction pathway.

As discussed previously (4), cultured prostate cells respond poorly to selenomethionine (a commonly used selenium reagent) and only when it is present at supraphysiological levels in the medium. A plausible explanation is that prostate cells have a low capacity in metabolizing selenomethionine to methylselenol (CH_3SeH), which is believed to be the active species for selenium chemoprevention (5). This process normally takes place in the liver and kidney. For this reason, methylseleninic acid (CH_3SeO_2H, abbreviated to MSA) was developed by Ip *et al.* (6) specifically for *in vitro* experiments. Once taken up by cells, MSA is readily reduced by glutathione and NADPH to methylselenol (which is rather unstable in itself) via a non-enzymatic reaction. The cellular and molecular responses of prostate cells to physiological concentrations of MSA have been documented in a number of publications (4, 7–10). Thus, we believe we have the right tool to study the effect of selenium on AR signaling.

Materials and Methods

Selenium Reagent, Prostate Cell Culture, and Cell Growth Analysis. MSA was synthesized as described previously (6). The LNCaP human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (11). In some experiments, cells were cultured in an androgen-defined condition by using charcoal-stripped FBS in the presence of 10 nM R1881 (a potent synthetic androgen). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed 24, 48, or 72 h after MSA treatment as described previously (11). For the quantitative determination of AR and PSA transcripts and proteins, cells were exposed to MSA for much shorter periods of time, usually 24 h or less. Total RNA and protein were isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA).

Real-Time Reverse Transcription-PCR. First-strand cDNA was synthesized from 100 ng of total RNA by SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. The PCR primers and TaqMan probes for β-actin, AR, and PSA were Assays-on-Demand products from Applied Biosystems. Two μl of first-strand cDNA were mixed with 25 μl of 2× Taqman Universal PCR Master Mix (Applied Biosystems) and 2.5 μl of 20× primers/probe mixture in a 50-μl final volume. Temperature cycling and real-time fluorescence measurement were performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: an initial incubation at 50°C for 2 min, then a denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

The relative quantitation of gene expression was performed using the comparative C_T ($\Delta\Delta C_T$) method (12). Briefly, the threshold cycle number (C_T) was obtained as the first cycle at which a statistically significant increase in fluorescence signal was detected. Data normalization was carried out by subtracting the C_T value of β-actin from that of the target gene. The $\Delta\Delta C_T$ was calculated as the difference of the normalized C_T values (ΔC_T) of the treatment

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Notes: Y. Dong and S. O. Lee contributed equally to this work.

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and control samples: $\Delta\Delta C_T = \Delta C_{T\ treatment} - \Delta C_{T\ control}$. Finally, $\Delta\Delta C_T$ was converted to fold of change by the following formula: Fold of change = $2^{-\Delta\Delta C_T}$.

Western Blot Analysis. Details of the procedure were described previously (4). Immunoreactive bands were quantitated by volume densitometry and normalized against either glyceraldehyde-3-phosphate dehydrogenase or α-actin. The following monoclonal antibodies were used (source): anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, CA), anti-α-actin (Sigma Chemical Co., St. Louis, MO), anti-AR (BD Transduction Laboratory, San Jose, CA), and anti-PSA (Santa Cruz Biotechnology, Santa Cruz, CA).

Transfection and Luciferase Assay. An aliquot of 3×10^5 cells was placed in a 6-well plate and then transfected with a total amount of 5 μg of DNA using Superfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Two different constructs were evaluated: the PSA promoter-luciferase reporter plasmid (13) and the androgen responsive element (ARE)-luciferase reporter plasmid (14). The total amount of plasmid DNA was normalized to 5 μg/well by the addition of empty plasmid. The DNA/liposome mixture was removed 3 h later, and cells were treated with different concentrations of MSA in the presence of 10 nM R1881. Cell extracts were obtained after 24 h, and luciferase activity was assayed using the Luciferase Assay System (Promega, Madison, WI). Protein concentration in cell extracts was determined by the Coomassie Plus protein assay kit (Pierce, Rockford, IL). Luciferase activities were normalized by the protein concentration of the sample. All transfection experiments were performed in triplicate wells and repeated at least four times.

Nuclear Lysate Preparation. Nuclear protein extract was prepared as described previously (15). Cells were harvested, washed with PBS twice, and resuspended in a hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% NP40] and incubated on ice for 10 min. Nuclei were precipitated with 3000 × *g* centrifugation at 4°C for 10 min. After washing once with the hypotonic buffer, the nuclei were lysed in a lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100] and incubated on ice for 30 min. The nuclear lysate was precleared by 20,000 × *g* centrifugation at 4°C for 15 min. Protein concentration was determined by the Coomassie Plus protein assay kit.

Electrophoretic Mobility Shift Assay (EMSA). A quantity of 20 μg of nuclear protein extract was incubated in a 20-μl solution containing 10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 μg/ml poly(deoxyinosinic-deoxycytidylic acid), and the radiolabeled double-stranded AR consensus binding motif 5′-CTAGAAGTCTGGTACAGGGT-GTTCTTTTTGCA-3′ (Santa Cruz Biotechnology). The protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25× Tris-borate EDTA at room temperature, and the results were autoradiographed. Quantitation of AR DNA-binding activity in the "protein-DNA" bandshift was measured using the Molecular Imager FX System (Bio-Rad, Hercules, CA). For the supershift experiment, 20 μg of cell extract protein were incubated with the monoclonal AR antibody (Santa Cruz Biotechnology) for 1 h at 4°C before incubation with the radiolabeled probe.

Results

MSA Inhibits LNCaP Cell Growth in a Dose- and Time-dependent Manner. Table 1 shows the results of the effect of MSA treatment on cell growth. The data are expressed as percentages of the untreated control. A concentration of 2.5 μM MSA produced essentially no change, even after 3 days of treatment. Increasing the concentration of MSA to 5 μM inhibited cell growth by about 25%, but the effect was not observed until the 72-h time point. The same magnitude of growth inhibition was observed at 24 h with 10 μM MSA, and by 72 h, there were 50% fewer cells compared with the untreated culture. The experiment therefore established the dose and time response to MSA with respect to growth inhibition. This information is important because the down-regulation of PSA and AR by selenium occurs well before the onset of growth inhibition (see below).

Table 1 *Effect of MSA on the accumulation of LNCaP cells*[a]

Treatment	Treatment duration (h)[b]		
	24	48	72
MSA (μM)			
2.5	102.5 ± 4.0	106.6 ± 6.2	102.5 ± 1.9
5	93.7 ± 3.1	96.4 ± 2.9	72.6 ± 1.9[c]
10	77.1 ± 8.4[c]	61.1 ± 1.7[c]	55.4 ± 3.7[c]

[a] As a percentage of untreated control.
[b] Results are expressed as mean ± SE (n = 4 independent experiments).
[c] Significantly different compared with the corresponding control value ($P < 0.05$).

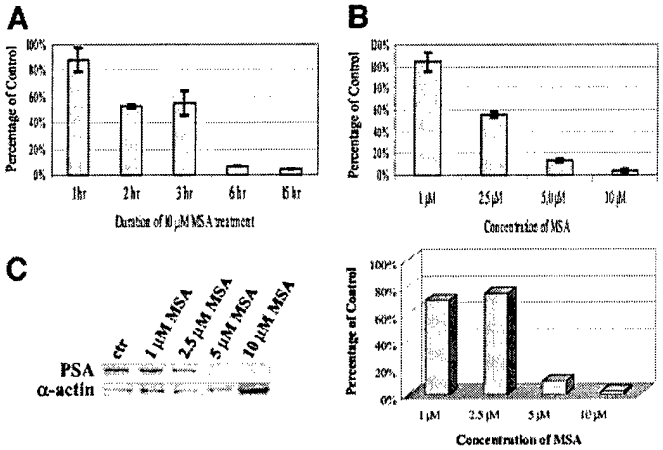

Fig. 1. Effect of MSA on PSA expression. *A* and *B*, changes in PSA mRNA, as determined by quantitative RT-PCR, as a function of time of treatment with MSA or as a function of MSA concentration. With the exception of the 1-h and the 1 μM MSA data points, the remaining data points are significantly different ($P < 0.01$) from the control, which is set as 100%. *Bars*, SE. *C*, Western blot data of changes in PSA protein level as a function of different concentrations of MSA (*left side*) and normalized quantitative changes compared with the control value of 100% (*right side*).

MSA Suppresses PSA mRNA and Protein Expression in LNCaP Cells. The modulation of PSA mRNA by MSA was assessed quantitatively by real-time RT-PCR. Cells were treated with 10 μM MSA for various lengths of time; the PSA results are shown in Fig. 1*A*. A marked decrease in PSA mRNA was detected as early as 2 h after exposure to MSA; the mRNA level dropped to <10% of the control value by 6 and 15 h. As shown in Fig. 1*B*, the depression of PSA mRNA was dependent on the concentration of MSA in the range between 2.5 and 10 μM; the assay was performed after exposure to MSA for 15 h. As little as 2.5 μM MSA reduced PSA mRNA level by 40%. This level of MSA had no effect on cell growth. Even with 10 μM MSA, the near complete elimination of PSA mRNA expression occurred before there was any detectable change in growth. The decrease in PSA protein level by MSA at 15 h, as determined by Western blot analysis, is shown in Fig. 1*C, left panel*. The right panel shows the normalized quantitative changes compared with the control value of 100%. Small decreases in PSA protein were evident with 1 or 2.5 μM MSA. At 5 or 10 μM MSA, the level of PSA protein became very low or hardly detectable. The experiments described in Fig. 1 were done with cells cultured in 10% FBS. In addition, we carried out another set of experiments with cells cultured in charcoal-stripped FBS containing 10 nM R1881 (a potent synthetic androgen). The down-regulation of PSA mRNA by MSA, as a function of dose and time, was qualitatively similar to that observed with the FBS culture (data not shown).

MSA Suppresses AR mRNA and Protein Expression in LNCaP Cells. The expression of PSA is known to be regulated by AR, which is a ligand-activated transcription factor. Our next step was to investigate the expression of AR mRNA in response to MSA by real-time RT-PCR. Fig. 2*A* shows the time course of response to 10 μM MSA. Within the first hour, there was a 50% decrease in AR mRNA. The transcript level continued to drop down to 20% or below with longer

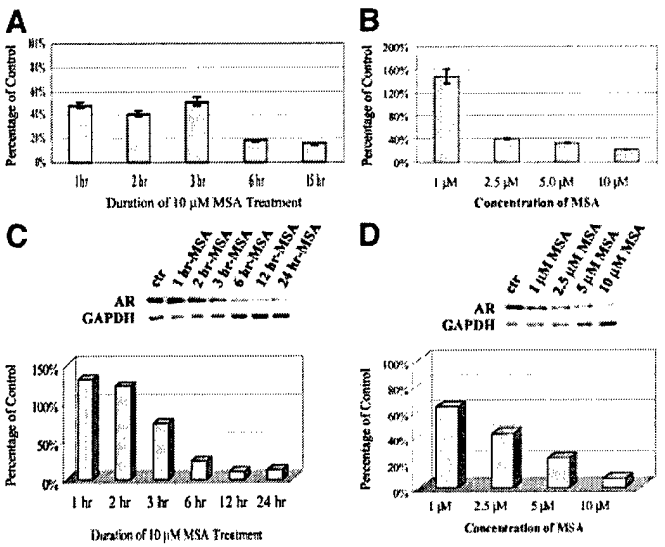

Fig. 2. Effect of MSA on AR expression. *A* and *B*, change in AR mRNA, as determined by quantitative RT-PCR, as a function of time of treatment with MSA or as a function of MSA concentration. All of the data are significantly different ($P < 0.01$) from the control, which is set as 100%. *Bars*, SE. *C*, Western blot data of changes in AR protein level as a function of time of treatment with 10 μM MSA. *D*, Western blot data of changes in AR protein level as a function of different concentrations of MSA. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

treatment with MSA. The dose response to MSA is shown in Fig. 2*B*; these assays were done at the 15-h time point. Interestingly, 1 μM MSA actually increased slightly the level of AR mRNA. However, raising the concentration of MSA to 2.5 μM or above caused a very significant depression of the AR transcript to 40% or less of the control value. We next examined AR protein expression in response to 10 μM MSA. As shown in Fig. 2*C*, MSA down-regulated AR protein level progressively as a function of time over a period of 24 h. Initially, the reduction in protein appeared to lag behind the reduction in transcript by at least 2–3 h. The delay in response might be reflective of the time needed for protein turnover. Fig. 2*D* shows the effect of different concentrations of MSA on expression of the AR protein. MSA produced a graded suppression of the AR protein in a dose-dependent manner. In general, the changes in protein level were consistent with the real-time RT-PCR results with the exception of the 1 μM MSA data point.

MSA Inhibits AR *trans*-Activating Activity. LNCaP cells have a mutant but functional AR. In an attempt to determine the ability of MSA to interfere with AR *trans*-activating activity, we transiently transfected LNCaP cells with an expression vector for the wild-type AR and the PSA promoter-luciferase reporter plasmid. This region of the PSA regulatory element contains the promoter and enhancer and has been demonstrated to be responsive to androgen stimulation (13). As shown in Fig. 3*A*, MSA inhibited the luciferase reporter in a dose-dependent manner. Thus, the PSA promoter activity was decreased by 50, 67, 93, or 96% in the presence of 1, 2, 5, or 10 μM MSA, respectively.

Activated AR exerts its function by binding to the ARE site. Because the PSA promoter contains many regulatory elements in addition to the ARE, one could argue that the decrease in PSA promoter activity might not necessarily be attributable to a change in AR *trans*-activating activity. To address this issue, we transiently transfected LNCaP cells with an expression vector for the wild-type AR and the ARE-luciferase reporter plasmid. This construct contains three repeats of the ARE region ligated in tandem to the luciferase reporter (14). As shown in Fig. 3*B*, the ARE-luciferase activity was inhibited by 50, 60, or 75% in the presence of 1, 2, or 5 μM MSA, respectively. The result obtained with 10 μM MSA was similar to that with 5 μM MSA.

MSA Decreases Binding of AR to ARE. To determine whether MSA might reduce the DNA binding activity of the AR protein to the ARE, we performed EMSA using radiolabeled oligonucleotides of the ARE with nuclear extract from LNCaP cells treated for 30 min with various concentrations of MSA. As shown in Fig. 4, *A* and *B*, a decrease in AR-ARE complex formation was evident with MSA treatment compared with the untreated control. We can rule out the reduced availability of the AR protein as a contributing factor, because there was no change in AR protein after only 30 min of

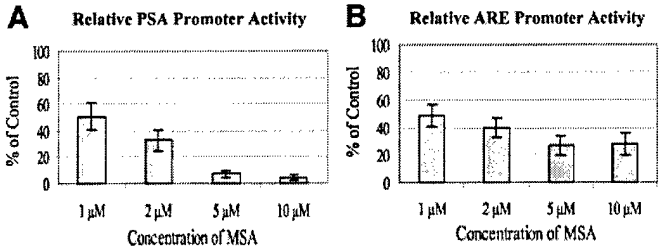

Fig. 3. Effect of MSA on PSA promoter activity (*A*) and ARE promoter activity (*B*). The cells were cultured in charcoal-stripped FBS containing 10 nM R1881. The results are expressed as percentages of untreated control. All of the data points are significantly different ($P < 0.01$) from the control value. *Bars*, SE.

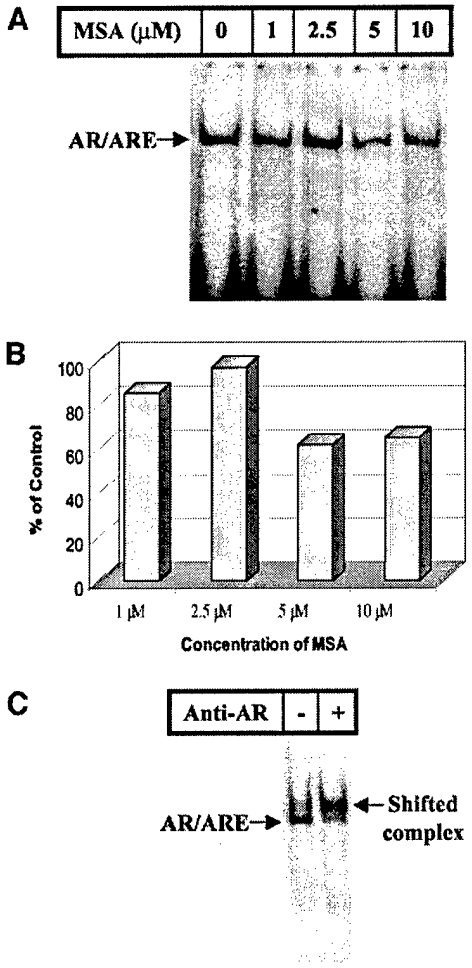

Fig. 4. *A*, EMSA results of AR binding to ARE as a function of different concentrations of MSA. *B*, quantitative determination of the EMSA results. *C*, supershift of the AR/ARE complex with antibody against AR.

treatment with MSA (see Fig. 2*C*). The specificity of the AR-ARE complex was demonstrated by the supershift assay using an antibody against AR (Fig. 4*C*).

Discussion

This report is the first to show that a selenium metabolite is able to down-regulate the expression of PSA in human prostate cancer cells via a mechanism involving disruption of the androgen signal transduction pathway. On the basis of the information from this study, selenium decreases AR transcript and protein and inhibits AR *trans*-activating activity. Selenium can also diminish the binding of AR to the ARE site. However, we cannot at this time distinguish whether this is attributable to a block in nuclear translocation of the activated AR or a physical interference of AR association with the ARE through modulation of other co-regulators. These various possibilities will be investigated systematically in the future. It is noteworthy that the reduction in AR and PSA expression occurs at least 20 h before any significant decrease in cell number. This kind of bellwether change at the molecular level might be one of the causes underlying the sensitivity of prostate cells to selenium treatment.

In a recent paper, Bhamre *et al.* (16) reported that although supraphysiological levels of selenomethionine inhibited LNCaP cell growth, selenomethionine did not specifically affect the production of PSA when the results were normalized to the decreased number of viable cells. As explained in the "Introduction," selenomethionine is not a suitable selenium reagent for cell biology studies *in vitro*, because it is poorly metabolized by cultured epithelial cells to the active monomethylated intermediate. Not surprisingly, many cellular and molecular events that are normally sensitive to modulation by physiological levels of MSA (4, 7–11) respond very sluggishly to selenomethionine, and only when it is present at excessively high levels in the medium. Thus, the discrepancy between our study and that of Bhamre *et al.* (16) can be reconciled by the differences in biochemical reactivity between MSA and selenomethionine.

The clonal expansion of prostate cancer at the early stage is mostly dependent on androgen stimulation. A selenium intervention strategy aimed at dampening the amplitude of androgen signaling could be helpful for controlling prostate cancer in high-risk men. PSA is a well-accepted diagnostic and prognostic biomarker of prostate cancer progression. The down-regulation of PSA by selenium therefore has significant clinical implication. In patients treated with selenium, the monitoring of PSA in the circulation could potentially be evaluated as a barometer to gauge the efficacy of intervention. The benefit might also be extended to the prevention of relapses after endocrine therapy. Recurrent prostate cancer is generally hormone refractory, although the expression of AR is maintained regardless of the clinical stage of the disease (17, 18). The fact that PSA continues to be produced by the pathologically advanced cancer suggests that the AR signal transduction pathway is still intact. Several hypotheses have been proposed to explain this phenomenon. Mutations of the AR may enable cells to be sensitized by very low levels of androgens, or perhaps even by non-androgen steroids (19). Alternatively, the receptor may become promiscuous and can be activated by non-steroidal growth factors and cytokines (20). Prostate cancer may also adapt to androgen deprivation by increasing the expression of AR through gene amplification (21, 22). We have developed a LNCaP subline that is not responsive to androgen but is capable of producing a copious amount of PSA. We are planning to use this cell model to further investigate the role of selenium in AR function when the presence of androgen is no longer required.

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Selenium Countermands the Expression of Androgen-Regulated Genes and Other Targets Implicated in Prostate Carcinogenesis[1]

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Running title: Selenium suppresses prostate cancer genes

Key words: selenium, chemoprevention, prostate carcinogenesis, androgen targets

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[6]The abbreviations used in the paper are: AR, androgen receptor; ARE, androgen responsive element; MSA, methylseleninic acid; PSA, prostate specific antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

Abstract

A previous trial showed that selenium supplementation significantly reduced the incidence of prostate cancer. Little is known about whether selenium might interfere with androgen action or modulate the expression of genes implicated in prostate carcinogenesis. The present study examined the cellular and molecular effects of selenium in the androgen-responsive LNCaP human prostate cancer cells. Physiological concentrations of selenium, in the form of methylseleninic acid, produced a dose- and time-dependent inhibition of growth with this cell line. An arrest at G_0/G_1 phase and a concomitant delay of passage to S phase were observed at 24 h. A marked decrease in the transcript and protein level of androgen receptor (AR) and prostate-specific antigen (a key target gene of AR) was detected as early as 6 h, suggesting that the interference of androgen signaling by selenium is not a consequence of growth arrest. In order to identify other AR-targets responsive to selenium modulation, gene expression profiling was carried out with a 3K cDNA microarray at 3, 6, 12, 24, 36 or 48 h of selenium treatment. The analysis showed that selenium was able to countermand the expression of a subset of 12 additional AR-regulated genes. These genes contain the androgen responsive element (ARE) motifs in their promoters. The information suggests that selenium might be capable of directly modulating the primary targets of AR signaling. As a follow-up to the above analysis, we also examined whether selenium could favorably alter the expression of putative prostate oncogenes, tumor-suppressor genes, or genes implicated in the transformation to androgen-independency. A total of 12 genes were identified that may provide new clues in elucidating the mechanism of action of selenium chemoprevention of prostate cancer.

Introduction

Supplementation with a nutritional dose of selenium was found to reduce prostate cancer incidence by 50% in a previous randomized, placebo-controlled cancer prevention trial (1,2). Prostate cancer was actually a secondary endpoint in this study, which was designed originally to evaluate the effect of selenium on non-melanoma skin cancer. Since men accounted for a sizable proportion of the cohort (974 of a total of 1,312), there was sufficient power for the analysis of the changes in prostate cancer risk. When the prostate cancer data were further stratified, there was evidence of a greater reduction in risk from selenium supplementation among men who had a low baseline blood selenium and low circulating PSA^6 levels (2). It has been well documented that early stage prostate cancer is mostly responsive to androgen stimulation. The inference that the protective effect of selenium might be more pronounced in early stage prostate cancer, as reflected by low PSA secretion, lends support to the idea that selenium might affect the expression of androgen-regulated genes which are important to the etiology of prostate cancer.

Recently, we reported that in the androgen-unresponsive PC-3 human prostate cancer cells, a selenium metabolite, in the form of methylseleninic acid or MSA, blocked cell cycle progression at multiple transition points and induced apoptotic cell death (3). In the present study, we aimed to determine whether androgen-responsive prostate cancer cells are equally sensitive to MSA, and whether the expression of androgen-regulated genes is amenable to challenge by treating cells with selenium. These questions are of significant clinical implication because a positive answer would provide a rational justification for selenium intervention in middle-aged men who are more likely to develop prostate cancer of the androgen-responsive phenotype. The LNCaP prostate cancer cells were used in our experiments since they have a functional androgen receptor (albeit mutated) and are known to be androgen-responsive.

Microarray analysis is increasingly being used to identify molecular targets of chemoprevention. We had successes with the Affymetrix 12K-gene oligonucleotide array in identifying a spectrum of signaling and effector targets of selenium in PC-3 cells (3). This time we used a 3K cDNA array; the smaller array is expected to improve the sensitivity of the assay, although the advantage is compromised by the reduced size of the dataset. There are three recent publications which are of special interest to our research on selenium chemoprevention of prostate cancer. All three studies applied the microarray technology to profile gene expression changes which are either related to androgen stimulation in LNCaP cells (4), prostate carcinogenesis (5), or the transformation from androgen-sensitive to androgen-independent phenotype (6). The above references provide the information source for the identification of a subset of genes of which the expression could be modulated by selenium in such a way that might be suggestive of a decrease in prostate cancer risk. In addition to generating new clues for the mechanism of action of selenium in prostate cancer chemoprevention, these genes could serve as potential biomarkers for evaluating the efficacy of selenium intervention.

Materials and Methods

Selenium reagent, prostate cell line, MTT cell growth assay, and cell cycle analysis. MSA was synthesized as previously described (7). The LNCaP human prostate cancer cell line was obtained from ATCC (Manassas, VA). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The MTT assay and cell cycle analysis were performed as described in our previous publication (3). Synchronized cells were used in cell cycle and all molecular analyses including microarray, real-time RT-PCR, Northern and Western blots. Synchronization was achieved by starvation in serum-free medium (3).

Apoptosis detection by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nicked end labeling). At 48 h after seeding, cells were exposed to 10 μM MSA for 48 or 72 h. Adherent cells harvested by mild trypsinization were pooled with detached cells. Cells were then cytospinned onto a silanized microscope slide and fixed in 1% paraformaldehyde. Apoptosis was detected by the TUNEL method using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Color pictures were taken with a camera mounted on top of a microscope under a 40X objective. All hard copy images were scored by an observer blinded to the identity of the sample in order to avoid bias. The quantification of apoptotic cells was calculated as a percentage of the total number of cells evaluated (> 600 cells/sample).

cDNA microarray analysis. LNCaP cells were plated at a density of 10^4 cells/cm^2 in 15-cm culture dishes. After exposure to 10 μM MSA for 3, 6, 12, 24, 36, or 48 h, total RNA and protein were isolated using TRIzol (Invitrogen, Carlsbad, CA). The experiment was repeated twice, and the total RNA collected from the three experiments was pooled and subjected to

microarray analysis. A 3K human cDNA microarray printed at the Microarray and Genomics Core Facility at Roswell Park Cancer Institute was used in this study. Each gene on this array was spotted in triplicate. Probe generation and array hybridization were conducted according to a protocol developed by the Core Facility[7]. The hybridization signals were captured using an Affymetrix 428 array scanner (Affymetrix, Santa Clara, CA), and analyzed using the ImaGene software (BioDiscovery, Inc., Marina Del Ray, CA). Poor quality spots, along with spots with signal levels indistinguishable from the background, were disgarded as bad spots. The extracted image data were then subjected to data analysis including background subtraction, data normalization, ratio calculation, and statistical analysis of replicate spots. Data analysis was conducted using the ImaGene (BioDiscovery, Inc., Marina Del Ray, CA) and the GeneTraffic software (Iobion Informatics LLC, La Jolla, CA), the statistical package R, and in-house PERL scripts. In order to control for the noise introduced by the fluorescent dyes, Cy3 and Cy5, each array experiment was performed twice using reciprocal labeling, and the signal ratios from these two experiments were averaged. A treatment to control signal ratio of ≥ 2 or ≤ 0.5 was chosen as the criteria for induction or repression, respectively. These threshold values are commonly used in the literature for microarray expression analysis (8-10). Hierarchical clustering analysis was performed using the Hierarchical Clustering Explorer software from the University of Maryland .

Northern and Western blot analysis. Northern analysis was performed with 25 μg of RNA samples. Northern blotting and hybridization were conducted using standard procedures. Relative sample intensities in autoradiographs were quantitated by volume densitometry using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and normalized to 18S rRNA. AR, PSA, CDC6, MRP4, ATF3, and c-myc cDNA clones were obtained from Invitrogen

[7] http://microarrays.roswellpark.org/Protocols

(Carlsbad, CA), and used to prepare probes for Northern hybridization. Details of the procedure for Western blot analysis were described previously (3). Immunoreactive bands were quantitated by volume densitometry and normalized to GAPDH. The following monoclonal antibodies were used in this study (source): anti-GAPDH (Chemicon, Temecula, CA), anti-AR, -CDK2 (BD Transduction Laboratory, San Jose, CA), and anti-PSA, -CDC6 (Lab Vision, Fremont, CA). Polyclonal antibody to ATF3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Real-time RT-PCR. First-strand cDNA was synthesized from 100 ng total RNA using the Superscript™ II reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The PCR primers and TaqMan® probes for β-actin, AR and PSA were Assays-on-Demand® products from Applied Biosystems (Foster City, CA). Two μl of first-strand cDNA was mixed with 25 μl of 2X Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 2.5 μl of 20X primers/probe mixture in a 50 μl final volume. Temperature cycling and real-time fluorescence measurement were performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: an initial incubation at 50°C for 2 min, then a denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

The relative quantitation of gene expression was performed using the comparative C_T ($\Delta\Delta C_T$) method (11). Briefly, the threshold cycle number (C_T) was obtained as the first cycle at which a statistically significant increase in fluorescence signal was detected. Data normalization was carried out by subtracting the C_T value of β-actin from that of the target gene. The $\Delta\Delta C_T$ was calculated as the difference of the normalized C_T values (ΔC_T) of the treatment and control samples: $\Delta\Delta C_T = \Delta C_{T\ treatment} - \Delta C_{T\ control}$. Finally, $\Delta\Delta C_T$ was converted to fold of change by the following formula: Fold of change $= 2^{-\Delta\Delta C_T}$.

Extraction of microarray data from the literature for comparison of gene expression changes. The microarray datasets generated by DePrimo *et al.* (4) and Dhanasekaran *et al.* (5) were downloaded from their respective websites. The data were filtered to include only the expression changes that were reported to be significant. The dataset generated by Karan *et al.* (6) is relatively small, it was input manually on the Excel spreadsheet. In the microarray data output, genes are usually identified by the accession numbers of the transcripts. The same gene included in the microarrays from various sources could have different accession numbers. In order to compare the gene expression pattern of our array with that of the three published datasets, we used the accession numbers to query the Unigene database at the National Center for Biotechnology Information (NCBI) to map the transcripts to their respective Unigene clusters. Unigene cluster is a unique representation of a gene. In the few cases that multiple transcripts in the array were mapped to the same cluster, the signal ratios of these transcripts were averaged to obtain a mean expression ratio. Since our array experiments and the studies by DePrimo *et al.* (4) and Dhanasekaran *et al.* (5) were conducted at several time points or with multiple samples, we have to make the decision call categorizing the pattern of gene expression changes across all the time points or samples, e.g., up-regulated, down-regulated, no change, or mixed. Based on the decision calls, genes that were changed in the same or opposite directions were identified. The above analyses were performed with in-house PERL programs.

Identification of androgen-responsive element (ARE) motif. The reference sequence identifiers (refSeqID) of a subset of androgen-regulated genes were retrieved from the LocusLink database at NCBI and used to query the human genome assembly at the University of California at Santa Cruz (version hg16) in order to obtain the mapping information of each gene. Based on this information, 5 kb of genomic sequence upstream of each transcript was retrieved.

The consensus ARE motif, AGAACANNNTGTTCT, was obtained from the TRANFAC database (version 6.0 public). The promoter sequences were analyzed for the presence of putative AREs with custom PERL script using regular expression. A maximum of 3 mismatches (≥75% identity) were allowed.

Statistical analysis. The Students 2-tailed *t*-test was used to determine significant differences between treatment and control values, and $P < 0.05$ was considered statistically significant.

Results

MSA inhibits LNCaP cell growth, blocks cell cycle progression and induces apoptotic cell death. MSA at a concentration of 5 μM decreased cell growth by about 25% after 72 h (data not shown). Raising the concentration to 10 μM produced a more rapid response and a greater degree of inhibition as a function of time. Thus, total cell number was reduced by 25% or 50%, respectively, after 24 h or 72 h of exposure to 10 μM of MSA. For cell cycle analysis by flow cytometry, synchronized cells were treated with 10 μM MSA for 24, 32 or 48 h. The results in Fig. 1 show that a block at G_0/G_1 phase (accompanied by a decreased passage to S phase) was observed at 24 h post-MSA treatment. The block persisted for at least eight more hours (significant difference at the 32 h time point), but seemed to relax gradually when the culture was maintained for a longer period (the difference was no longer statistically significant at the 48 h time point). At 48 h post-MSA treatment, an increase in apoptotic cell death was evident (Fig. 2). The induction of apoptosis by MSA continued to escalate with time. The experiment described in Fig. 2 was carried out using the TUNEL assay. We had originally tried the annexin V staining method to assess apoptotic cell death by flow cytometric analysis (which we had successfully done with PC-3 cells). We found that this method produced an unusually high estimation of apoptosis, in the neighborhood of 20-25%, even for the untreated LNCaP cells. Visual inspection of these same cells under the microscope for chromatin condensation certainly did not support the results of the annexin V assay. Although annexin V staining is commonly used for the detection of apoptosis, we have since confirmed that it is not at all suitable for LNCaP cells for some unknown reasons (similar information was corroborated by Junxuan Lu at the University of Minnesota, personal communication).

Microarray analysis of cells treated with MSA. In order to identify potential targets of MSA in LNCaP cells, we examined the change in gene expression profile using a 3K human cDNA array at 3, 6, 12, 24, 36 and 48 h post-MSA treatment. A hierarchical clustering algorithm was applied to group genes according to similarities in their expression pattern across time points. The clustering analysis of the expression pattern of 762 MSA-responsive genes is shown in Fig. 3. The branch points in the dendogram correspond to each gene, and the distance between the points reflects the degree of relatedness. Red and green squares represent up-regulation and down-regulation, respectively, relative to the corresponding control sample. Black squares indicate levels not significantly different from the control, and gray squares signify data of insufficient quality. The gene identities and the raw array data are available at our website[8]. Four distinct clusters emerged from this analysis. Clusters A and C are composed of genes with a gradual or a rapid increase in expression level, respectively. Clusters B and D represent the group of genes with a rapid or gradual reduction in expression level, respectively.

MSA down-regulates AR and PSA expression. Androgen receptor (AR) is a ligand-activated transcription factor (12). The availability of AR is likely to play a role in the transcriptional program activated by androgen. The microarray data of the effect of MSA on AR are highlighted in particular and shown in Table 1. The values are expressed as treatment to control signal ratio. Thus a value of ≤ 0.5 denotes a significant down-regulation by MSA. The decrease of AR expression was seen as early as 6 h. The level of AR transcript reached a nadir at 12 h, but gradually rebounded with time, although it was still slightly down by 48 h. Fig. 4A and 4B show the marked reduction of AR by Northern blot or real-time RT-PCR analysis,

[8] http://www.roswellpark.org/Dong_Ip_Se_microarray/

respectively, at 6, 12 or 24 h of MSA treatment. The decreases in AR message level were accompanied by parallel decreases in protein level, as shown by the Western blot in Fig. 4C.

PSA is probably the most celebrated AR-regulated gene from a clinical standpoint because it is a well accepted marker for the diagnosis and prognosis of prostate cancer. A down-regulation of AR by MSA would be expected to lead to decreases in PSA expression. Fig. 5A and 5B show the Northern blot and real-time RT-PCR data of PSA. Robust decreases in PSA transcript were observed at 6, 12 and 24 h after treatment with MSA. These changes were accompanied by a marked depression in PSA protein level, as shown by the Western blot in Fig. 5C.

It is noteworthy to point out that the AR and PSA data were obtained from synchronized cells. After allowing cells to attach to the bottom of the culture dishes, synchronization was achieved by placing them in a serum-free medium for 48 h. More relevant to the interpretation of our data, this procedure removed androgen from the culture. Upon the addition of serum to relieve cells from starvation, the re-introduction to androgen stimulation might have contributed to the differential levels of AR and PSA at the early (3 h) and later ($\geq$ 6 h) time points in the control samples. These cells had gear up the androgen signaling machinery for the production of PSA, thus accounting for the low expression of both AR and PSA at 3 h. By 6 h, there was a healthy recovery of AR and PSA in control cells (Fig. 4 and 5). The observation that MSA can markedly depress AR and PSA expression at 6 h and beyond suggests that MSA has the capability of blocking *de novo* synthesis of AR and PSA. Furthermore, no perturbation of cell cycling was detected at 6 h post-MSA treatment, when there was already a strong suppression of both AR and PSA. Therefore, the down-regulation of PSA (and other androgen-responsive genes by extension) is unlikely to be related to some non-specific consequence of growth arrest.

MSA countermands the expression of a host of androgen-regulated genes. From our array analysis, we were able to identify an additional 12 androgen-regulated genes of which the expression was altered by MSA (Table 2). These 12 genes are categorized as responsive to androgen by DePrimo *et al.* (4) based on their expression changes in LNCaP cells that have been treated with R1881 (a potent synthetic androgen). As shown in Table 2, the expression of the first nine genes was repressed by MSA; the same nine genes were reported by DePrimo *et al.* (4) to be up-regulated by androgen. The expression of the last three genes was induced by MSA; all three were down-regulated by androgen as described in the DePrimo paper (4). It is interesting to note that the expression changes of 11/12 genes (the exception was IκB-α) began to gain statistical significance after 6 h of MSA treatment, at a time when the suppression of AR was observed (Table 1). Thus, the changes in the transcript signal of this group of genes were consistent with their regulation by AR. The modulation of IκB-α by MSA was rather unusual for two reasons. First, the decrease in the expression of IκB occurred early (at 3 h), and preceded the significant down-regulation of AR. Second, a reduced level of IκB-α is counter-intuitive to the action of selenium, because selenium enhances apoptosis, and IκB-α inhibits the activity of NFκB, which blocks apoptosis. Nonetheless, the consistency of the IκB-α data raises a legitimate question regarding the role of IκB-α in mediating the effect of selenium.

In order to verify that all 12 genes are primary, and not secondary, targets of androgen, we studied the promoter regions for sequences homologous to the consensus ARE, 5'-AGAACANNNTGTTCT-3'. Using their respective reference sequences, we mapped these genes to the human genome assembly with in-house PERL programs. A 5 kb promoter sequence was retrieved for each of the 12 genes. As shown in Table 3, the promoter region of these genes contains a motif comprising of at least 12 of 15 nucleotides (80%) corresponding to the

consensus ARE. The high prevalence of ARE motif lends support to the notion that MSA might be able to countermand directly a subset of androgen-regulated genes.

We carried out Northern and/or Western analysis of CDC6 and MRP4 using TRIzol extract of cells treated with MSA (Fig. 6). The purpose was to pick two genes from Table 2 to confirm our array data. Decreases in CDC6 mRNA and protein, as well as decreases in MRP4 mRNA, were evident with MSA treatment at multiple time points. The potential involvement of CDC6, MRP4 and the other androgen-regulated genes in carcinogenesis is elaborated in the Discussion section.

MSA countermands the expression of genes implicated in prostate carcinogenesis and progression. An analysis of our microarray dataset showed that the expression of four putative prostate oncogenes was down-regulated by MSA (Table 4). There were, however, subtle differences in the kinetics of change. The decreases in c-myc and IGF-binding protein 3 occurred early, but seemed to recover gradually with time. In contrast, the decreases in topoisomerase II-α and clone 23620 mRNA were hardly significant at the beginning, but became more pronounced at the later time points. Table 4 also shows that the expression of three putative prostate tumor-suppressor genes was up-regulated by MSA. The increase in ATF3 was particularly striking, especially during the first 24 h of MSA treatment. The above classification of potential oncogenes and tumor-suppressor genes involved in prostate carcinogenesis is based on the gene expression profiling information provided by Dhanasekaran *et al.* in their recent publication (5).

From our array analysis, we found that MSA was able to down-regulate the expression of six genes implicated in the transition from androgen-dependent to –independent growth of prostate cancer (Table 5). These genes are so classified because they are deemed to be important

in the transformation of the early passage androgen-sensitive LNCaP cells to the late passage androgen-independent LNCaP C81 cells as described by Karan *et al.* (6). In general, their repression by MSA was steady; there were little data fluctuations across time points. The consistency provides a fair measure of confidence regarding the validity of our observation.

We carried out Northern and/or Western analysis of three genes in particular: ATF3, c-myc and CDK2 (Fig. 7). In cells treated with MSA, the increase in expression of ATF3 (mRNA and protein), and the decrease in expression of c-myc (mRNA) and CDK2 (protein) were consistent with the array data. The significance of some of the genes listed in Tables 4 and 5 is discussed in the next section.

Epilogue

As a concluding remark to the Results section, we want to point out that we could have incorporated a bigger list of genes in each of Tables 2, 4 and 5. In our microarray analysis, we routinely disgarded poor quality spots (see Methods) and excluded them from evaluation. We followed a very stringent criterion of not including anything with more than one missing data point in a set of six time points of analysis. Thus, there is only one missing data point (denoted as NA, not available) in all of Tables 2, 4 and 5. We believe this strict guideline of data filtering greatly improves the reliability of our findings.

Discussion

Menter *et al.* (13) have previously described the growth inhibitory effect of selenomethionine on LNCaP cells and reported an IC_{50} of well over 100 μM after 48 h of exposure. In our hands, it took 200 μM selenomethionine to duplicate approximately the growth inhibitory effect of 10 μM MSA in LNCaP cells (data not shown). Based on the studies with a variety of cancer cell models, MSA is at least 20X more potent than selenomethionine (14-19). The ability to use physiological levels of MSA to generate biological data is clearly critical to our research questions. With our array analysis, we have uncovered a number of potentially exciting clues regarding the chemopreventive action of selenium in prostate cancer. Presently, the spotlight is focused on the findings that selenium is able to modulate, in the opposite direction, the expression of a subset of androgen-regulated genes, as well as some genes that are implicated in prostate carcinogenesis and progression from androgen-dependency to -independency (refer to summary in Table 6). This is very preliminary information, and the value of this information can be enhanced only by additional systematic investigation of the new leads.

Androgen plays an important role not only in maintaining the function of the prostate, but also in promoting the development of prostate cancer (20). In addition to down-regulating AR mRNA and protein (Fig. 4), we recently showed that selenium is capable of suppressing the binding of AR to the ARE site in a gel shift assay, as well as inhibiting the trans-activation of AR in an ARE-luciferase construct reporter assay (21). The present results showed that a subset of 13 androgen-regulated genes were modulated in the opposite direction by selenium. This is admittedly a small fraction of all putative androgen-regulated genes identified by DePrimo *et al.* (4), even though the discrepancy could in part be accounted for by the fact that their dataset is

much larger than ours. In doing this kind of array comparison, two things in particular must be taken into consideration. First, the genes in the DePrimo file are not necessarily all direct targets of androgen (the presence of ARE motifs have not been confirmed). Second, it is well recognized that genes have multiple regulatory elements, both positive and negative, on their promoter region. Selenium is known to alter the expression of many transcription factors, co-activators and co-repressors (3). The ARE is but one of many regulatory elements controlling the transcription of androgen-responsive genes. Collectively, these factors may explain why selenium could countermand the expression of some, but not all androgen-regulated genes. The critical question is to determine whether this subset is important for selenium chemoprevention of androgen-dependent prostate cancer.

We previously described the down-regulation of AR and PSA expression in LNCaP cells treated with different doses of MSA (21). The experiments, however, were done with asynchronous cells. The kinetics of AR and PSA suppression seen in this condition was quite different than that seen in synchronized cells (Fig. 4 and 5). In asynchronous cell culture in which there was an abundance of AR and PSA when treatment with MSA began, the inhibitory response was immediate (1 h post MSA). In contrast, in synchronized cell culture in which there was very little AR and PSA to begin with, the response to MSA was primarily due to an inhibition of de novo synthesis as suggested by our data. Based on the information from the two sets of experiments, we believe that the turnover of AR and PSA can also be affected by MSA. Indeed, our preliminary evidence corroborates an increased degradation by MSA in the absence of new synthesis (data not shown).

A brief comment is in order regarding the genes which are modulated by selenium and shown in Tables 2, 4 and 5. Many of them are involved in cell proliferation, cell cycle control or

apoptotic cell death. These include ribosomal protein S6 kinase 2, CDC42 effector protein 3, occludin, CDC6, seladin-1, cyclin G2 (from Table 2), topoisomerase II-α, ATF3 (from Table 4), cdk4, cdk2, and cyclin B1 (from Table 5). Selenium modulates this group of genes in a way that is consistent with the ability of selenium to inhibit cell growth, block cell cycle progression, and stimulate apoptotic cell death. Obviously it would be desirable to be able to confirm the expression changes of all 24 genes by Western or Northern analyses. For various reasons, one has to face reality in making choices. We picked five genes for confirmation and were successful with our effort (Fig. 6 and 7). These genes (c-myc, CDK2, CDC6, ATF3 and MRP4) are of special interest to us and are therefore highlighted briefly below.

The c-myc gene appears in both Tables 4 and 5. Several studies have reported the amplification and/or over-expression of c-myc in prostate cancer (22-24). Lactate dehydrogenase A (from Table 5), which is known to participate in anaerobic glycolysis, is frequently over-expressed in many cancers, including prostate cancer (25,26). It is a target of c-myc and is necessary for the malignant transformation by c-myc (27,28). Consistent with a down-regulation of c-myc by MSA, the expression of lactate dehydrogenase A is reduced as well. CDK2 and CDC6 are positive regulators of cell cycle progression. As elaborated in our previous publications with other cell lines (3,17), we believe that the down-regulation of CDK2 is critically important in mediating the growth inhibitory effect of selenium. Likewise, a reduced expression of CDC6 would also be consistent with a $G_1 \rightarrow S$ block by selenium (Fig. 1), since CDC6 is required for DNA replication (29). ATF3 is a direct target of p53, it is known to be associated with enhanced caspase activation and apoptosis (30,31). We have recently reported that treatment of PC-3 cells with selenium resulted in the activation of a number of initiator and executioner caspases (18). The role of ATF3 in mediating the apoptotic effect of selenium is

currently under investigation. The multi-drug resistant associated protein 4 (MRP4) is involved in the energy-dependent efflux of a variety of cytotoxic drugs (32). Therefore, a down-regulation of MRP4 by selenium could potentially lead to increased drug retention. The recent finding by Cao *et al.* at Roswell Park (33) that selenium is able to selectively modulate the therapeutic efficacy of a number of anticancer drugs has prompted an ongoing collaboration between the two groups of investigators.

Both of our selenium 12K Affymetrix GeneChip PC-3 file and the 3K cDNA microarray LNCaP file have been put on the website[8]. We found that 10 of the total of 12 genes included in Tables 4 and 5 were modulated by selenium in an identical manner in PC-3 cells as in LNCaP cells. Such a high degree of concordance (83%) between the two cell lines suggests that the list of genes that we pulled out from the microarray data is unlikely due to chance occurrences. The remaining two genes of which the expression was modulated by selenium in opposite direction in the two cell lines include c-myc and lactate dehydrogenase A. These genes might be critical in mediating the effect of selenium in blocking prostate cancer progression from androgen-dependency to –independency. The role of c-myc in selenium chemoprevention deserves further attention.

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Figure Legends

Fig. 1. Cell cycle distribution in LNCaP cells treated with MSA. Results are expressed as mean ± SE (n=3). *, statistically significant ($P<0.05$) compared to untreated control.

Fig. 2. Quantitation of apoptotic cell death by TUNEL assay in LNCaP cells treated with MSA. Results are expressed as mean ±SE (n=3). *, statistically significant increases ($P<0.05$).

Fig. 3. Hierarchical clustering analysis of MSA-responsive genes.

Fig. 4. Decreases of AR expression in MSA-treated cells. Panel A, Northern analysis. Panel B, Western analysis. Panel C, real time RT-PCR quantitation. *, statistically significant ($P<0.001$) compared to untreated control.

Fig. 5. Decreases of PSA expression in MSA-treated cells. Panel A, Northern analysis. Panel B, Western analysis. Panel C, real time RT-PCR quantitation. *, statistically significant ($P<0.001$) compared to untreated control.

Fig. 6. Decreases of CDC6 (Panel A) and MRP4 (Panel B) expression in MSA-treated cells as determined by Northern or Western analysis.

Fig. 7. Modulation of ATF3 (Panel A), c-myc (Panel B) and CDK2 (Panel C) expression in MSA-treated cells as determined by Northern or Western analysis.

Table 1. Repression of AR by MSA – microarray data*

Gene Name	Accession Number	Time Points (h)					
		3	6	12	24	36	48
AR	**AI659563**	0.8	0.4	0.2	0.3	0.7	0.8

*Expressed as treatment to control signal ratio. Values ≤0.5 denote significant down-regulation.

Table 2. MSA modulates the expression of androgen-regulated genes*

Gene Name	Accession Number	Time Points (h)					
		3	6	12	24	36	48
CDC6	H59204	1.3	1.2	1.0	0.4	0.2	0.2
MRP-4	AA165678	0.9	0.6	0.3	0.4	0.5	0.5
ribosomal protein S6 kinase 2	H55921	1.2	1.5	1.3	0.9	0.6	0.4
N-acetylglucosamine-phosphate mutase	AA001870	0.9	0.5	0.4	0.5	0.6	0.7
fatty-acid-Coenzyme A ligase	N98509	1.3	1.7	0.9	0.3	0.4	0.3
CDC42 effector protein 3	AA213816	1.2	0.9	0.4	0.4	0.4	0.5
IκB-α	W56300	0.4	0.3	0.4	0.3	0.6	0.9
kinesin-like 4	AA430503	0.7	0.7	0.6	0.5	0.6	0.6
seladin-1	AA482228	1.0	0.9	0.5	0.5	0.7	0.6
Ral guanine nucleotide exchange factor RalGPS1A	AA402863	1.2	1.6	NA	2.3	1.7	2.0
occludin	H94471	1.1	1.4	0.9	1.6	2.3	2.5
cyclin G2	AA489647	1.1	1.5	1.7	2.8	2.2	1.5

*Expressed as treatment to control signal ratio. Values ≥2 and ≤0.5 denote significant up-regulation and down-regulation, respectively. NA, not available.

Table 3. ARE motifs in the promoter region of the androgen-regulated genes that are modulated by MSA*

Gene Name	Accession Number	ARE sequence	Position	% of Match
CDC6	H59204	ATAACACCAGGTTCA	-1178	80
		AGACCAGTCTGTCCA	-2954	80
		AGAACATAATATTCT	-719	93
		TGAAAAGTCTGTTTT	-3050	80
MRP4	AA165678	AGAAAACCAAATTCT	-1186	80
		GGAACAAGATTTTGT	-1572	80
		ACAATAAAATGATCT	-1209	80
		ACAATAAAATGATCT	-1717	80
		AAAATATTGTGTTCC	-2971	80
		AGAAAAGAATGTTAA	-3352	80
		ACAAGATTTTGTTCA	-1569	80
ribosomal protein S6 kinase 2	H55921	AGAAATGCCTGTTCT	-4542	87
		AAAAAATCATTTTCT	-3143	80
N-acetylglucosamine-phosphate mutase	AA001870	GGAGGAATGTGTTCT	-2631	80
fatty-acid-Coenzyme A ligase	N98509	AGAAAAGAGTGGTTT	-1654	80
		GAAAGATAATGTTCT	-1392	80
		GGAACAGAATGGTGT	-1730	80
CDC42 effector protein 3	AA213816	AGGACATTCTCTTTT	-2680	80
		AGATCACAGGTTTCT	-2147	80
		AGAACAGAACGACCT	-4177	80
		TGAAGCAATTGTTCT	-3102	80
		AAAATACCTTTTTCT	-1158	80
IκB-α	W56300	AGAACAATGTGGTAC	-1886	80
		AGACCCTCTTGTTGT	-2653	80
kinesin-like 4	AA430503	TGAACAGCCTTTTCT	-2758	87
seladin-1	AA482228	AAAAAAAACTGTTCT	-3009	87
		AGAAGAACCTGGTAT	-910	80
		ATATCAAAATGTTAT	-1589	80
		AACAAACCCTGTTCT	-3370	80
		AGAACATCCTATTCC	-2886	87
		ATAAACAATTGTTCT	-2353	80
Ral guanine nucleotide exchange factor RalGPS1A	AA402863	AGAACACTTTCATCC	-3564	80
		AGTTCACGCCGTTCT	-1424	80
occludin	H94471	ACATCATAAAGTTCT	-4341	80
		AGAACATGTTTTTCC	-3312	87
cyclin G2	AA489647	CGAGCATTTAGTTCT	-4798	80
		TGATCAGTCTTTTCT	-2507	80

*ARE consensus: 5'-AGAACANNNTGTTCT-3'

Nucleotides identical to the ARE consensus are indicated in bold.

Table 4. MSA modulates the expression of genes implicated in prostate carcinogenesis*

Gene Name		Accession Number	Time Points (h)					
			3	6	12	24	36	48
Putative Oncogene	c-myc	AA464600	0.2	0.2	0.5	0.8	0.7	0.6
	IGF-binding protein 3	AA598601	0.5	0.5	0.6	0.4	1.1	2.5
	topoisomerase II-α	AA504348	0.7	0.6	0.4	0.1	0.1	0.2
	clone 23620 mRNA sequence	R40970	1.2	1.0	0.6	0.6	0.4	0.4
Putative Tumor Suppressor	glutaredoxin	AA291163	1.4	1.8	2.3	1.0	0.7	1.0
	ATF3	H21042	15.2	37.4	46.8	20.2	4.3	6.7
	ornithine aminotransferase	AA446820	1.3	2.1	4.6	3.0	1.3	1.2

*Expressed as treatment to control signal ratio. Values ≥2 and ≤0.5 denote significant up-regulation and down-regulation, respectively.

Table 5. MSA modulates the expression of genes implicated in the transition from androgen-dependent to –independent growth of prostate cancer*

Gene Name	Accession Number	Time Points (h)					
		3	6	12	24	36	48
c-myc	AA464600	0.2	0.2	0.5	0.8	0.7	0.6
cdk4	AA486208	0.9	0.8	0.7	0.5	0.5	0.5
cdk2	AI653017	1.1	1.0	0.6	0.2	0.3	0.3
cyclin B1	R46787	1.0	0.8	0.5	0.4	0.3	0.4
Arg/Ser-rich splicing factor 7	H54020	0.9	1.0	0.7	0.5	0.5	0.5
lactate dehydrogenase A	AA497029	0.9	0.9	0.8	0.4	0.6	0.5

*Expressed as treatment to control signal ratio. Values ≥2 and ≤0.5 denote significant up-regulation and down-regulation, respectively.

Table 6. MSA countermands the expression of androgen-regulated genes and other targets implicated in prostate carcinogenesis[1]

Gene Name	Expression Changes in Prostate Cancer Cells	Response to MSA
Androgen-regulated Genes[2]		
PSA	↑	↓
CDC6	↑	↓
MRP-4	↑	↓
ribosomal protein S6 kinase 2	↑	↓
N-acetylglucosamine-phosphate mutase	↑	↓
fatty-acid-Coenzyme A ligase	↑	↓
CDC42 effector protein 3	↑	↓
IκB-α	↑	↓
kinesin-like 4	↑	↓
seladin-1	↑	↓
Ral guanine nucleotide exchange factor RalGPS1A	↓	↑
occludin	↓	↑
cyclin G2	↓	↑
Oncogenes[3]		
c-myc	↑	↓
IGF-binding protein 3	↑	↓
topoisomerase II-α	↑	↓
clone 23620 mRNA sequence	↑	↓
Tumor Suppressor Genes[3]		
glutaredoxin	↓	↑
ATF3	↓	↑
ornithine aminotransferase	↓	↑
Transition to Androgen-independency[4]		
c-myc	↑	↓
cdk4	↑	↓
cdk2	↑	↓
cyclin B1	↑	↓
Arg/Ser-rich splicing factor 7	↑	↓
lactate dehydrogenase A	↑	↓

[1]↑ and ↓ denote significant up-regulation and down-regulation, respectively.
[2]DePrimo *et al.* (4). [3]Dhanasekaran *et al.* (5). [4]Karan *et al.* (6).