Expression profile of differentially-regulated genes during progression of androgen-independent growth in human prostate cancer cells

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Because of the heterogeneous nature of prostate cancer, identifying the molecular mechanisms involved during the transition from an androgen-sensitive to an androgen-independent phenotype is very complex. An LNCaP cell model that recapitulates prostate cancer progression, comprising early passage androgen-sensitive (LNCaP-C33) and late passage androgen-independent (LNCaP-C81) phenotypes, would help to provide a better understanding of such molecular events. In this study, we examined the genes expressed by LNCaP-C33 and LNCaP-C81 cells using cDNA microarrays containing 1176 known genes. This analysis demonstrated that 34 genes are up-regulated and eight genes are down-regulated in androgen-independent cells. Northern blot analysis confirmed the differences identified by microarrays on several candidate genes, including c-MYC, c-MYC purine-binding transcription factor (PuF), macrophage migration inhibitory factor (MIF), macrophage inhibitory cytokine-1 (MIC-1), lactate dehydrogenase-A (LDH-A), guanine nucleotide-binding protein Gi, α-1 subunit (NBP), cyclin dependent kinase-2 (CDK-2), prostate-specific membrane antigen (PSM), cyclin H (CCNH), 60S ribosomal protein L10 (RPL10), 60S ribosomal protein L32 (RPL32), and 40S ribosomal protein S16 (RPS16). These differentially-regulated genes are correlated with progression of human prostate cancer and may be of therapeutic relevance as well as an aid in understanding the molecular genetic events involved in the development of this disease’s hormone-refractory behavior.

Introduction

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer deaths in the male population of the United States. Progression of prostate cancer is characterized by a transition from an androgen-dependent to an androgen-independent phenotype, which determines the clinical outcome of the patient. The initial androgen-dependent stage of prostate cancer is organ-confined and is curable by means of surgery or other modalities. After the disease metastasizes to other organs and acquires androgen-independence, it frequently displays a fatal behavior. At the advanced stage of prostate cancer, most patients who are treated by androgen deprivation therapy exhibit a dramatic regression of the androgen-dependent cancer cells (1). Initially, 70–80% of the patients respond to the androgen ablation therapy, but the tumor eventually progresses to an androgen-independent stage, thereby resulting in a poor prognosis (2,3). Unfortunately, no effective alternative therapies are currently available for the hormone-refractory stage of this disease. Therefore, exploring the molecular mechanisms underlying prostate cancer’s progression to the androgen-independent stage is a key to introducing new therapies. In this regard, using DNA microarray technology to define alterations in gene expression associated with prostate cancer may be an efficient way of uncovering clues to the specific molecular derangements that contribute to its pathogenesis and thus of identifying potential targets for therapeutic intervention.

DNA microarray technology allows simultaneous determination of the expression level of thousands of genes. This approach has dramatic ramifications for deciphering and classifying gene expression changes in human cancers in a way that reflects the underlying molecular pathology or that anticipates their potential for progression or response to treatment. Over the last 5 years, DNA array analyses have largely identified genes that potentially play key roles in the malignant behavior of melanomas (4), embryonic stem cells (5), solid tumors of the breast (6), cervix (7), ovaries (8,9), colorectal carcinomas (10), and glioblastomas (11,12). Several recent studies have also suggested the classification of various cancers based on gene expression profiles established by DNA array analyses for improving the efficacy of treatment (13–15).

Information concerning the gene expression pattern in prostate cancer is limited due to the heterogeneous and multifocal behavior of the disease. Therefore, only a few recent studies have addressed this issue (16–20). Nevertheless, due to the complexity of prostate carcinomas, the results obtained from archival specimens would make it difficult to address the association between those gene expressions and hormone-refractory growth. To investigate the molecular mechanism of androgen-independent growth of prostate cancer cells, we have developed a unique in vitro cell model (21) derived from the LNCaP cell line, which was originally established from human prostate adenocarcinomas (22). This tumor cell model, which includes different LNCaP cells, closely resembles different stages of tumor progression and the acquisition of hormone-refractiveness in advanced prostate cancer patients (21,23). We previously observed that genetic alterations increased with decreased androgen-responsive behavior of the cells in this model. Furthermore, those genetic alterations identified in the cell model were seen in clinical specimens (24). Thus, this

Abbreviations: CCNH, cyclin H; CDK-2, cyclin dependent kinase-2; LDH-A, lactate dehydrogenase-A; MIC-1, macrophage inhibitory cytokine-1; MIF, macrophage migration inhibitory factor; NBP, guanine nucleotide-binding protein Gi, α-1 subunit; PSA, prostate-specific antigen; PSM, prostate-specific membrane antigen; PuF, purine-binding transcription factor; RPL10, 60S ribosomal protein L32, RPS16, 40S ribosomal protein S16.
cell model recapitulates prostate cancer progression from the androgen-sensitive to the androgen-independent stage.

In the present study, the LNCaP cell model was used to identify differentially-regulated genes associated with acquisition of the androgen-independent phenotype of the prostate tumor. A cDNA microarray gene filter containing 1176 known genes was employed to analyze genes expressed by early passage androgen-sensitive (C33) and late passage androgen-independent (C81) cells of the LNCaP cell model. We identified several genes that exhibit quantitative differences in their expression during progression of the androgen-independent phenotype. These genes appear to be helpful for understanding the biology of this disease and may prove useful in developing improved diagnosis and treatment of advanced stage prostate tumor.

Materials and methods

LNCaP cell model and cell culture

The LNCaP cell model used in the present study was developed by Lin et al. (21) and further characterized by Igawa et al. (23). Briefly, three stages of LNCaP cells during regular maintenance were designated as C33 (LNCaP passage number <33), C51 (LNCaP passage number between 45 and 70), and C81 (LNCaP passage number between 81 and 120). The cells in these three stages exhibit different androgen-responsiveness of growth stimulation; C33 cells are androgen-sensitive while C81 cells are androgen-independent. This cell model recapitulates the progression of human prostate tumor to the advanced hormone-refractory stage (21,23,24). In the study reported here, we utilized C33 and C81 cells of this LNCaP cell model to analyze the differentially-expressed genes during tumor progression. Cells were maintained in RPMI 1640 medium supplemented with 5% FBS, 1% glutamine, 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY) and passaged weekly by trypsinization.

RNA isolation

Total RNA was extracted from C33 and C81 stage LNCaP cells by using guanidine isothiocyanate–cesium chloride ultracentrifugation method (25). RNA concentration was measured spectrophotometrically, and its integrity was analyzed by electrophoresis on a formaldehyde agarose gel.

cDNA microarray hybridization

The mRNA expression profiles of C33 and C81 cells were surveyed using Atlas Human 1.2 cDNA arrays (Clontech Laboratories, Palo Alto, CA). For parallel hybridizations, total RNA (5 µg) from each cell population was reverse transcribed at 37°C for 90 min in a final reaction volume of 30 µl containing 5X first strand buffer, 0.1 M DTT, 20 mM dNTP, Reverse Transcriptase, oligo d(T) primers, and [32P]dCTP (10 µCi/ µl with a sp. act. of 3000 Ci/mmol). Probes were purified by chromatography with Nick™ Column (Pharmacia Biotech, NJ), and then denatured for 3 min in a boiling water-bath. Denatured radio-labeled probes were added to a 5 µl aliquot of MicroHyb hybridization solution (Research Genetics, Huntsville, AL) containing 5 µg of Cot-1 DNA and 5 µg of Poly d(A). This cDNA probe solution was applied to the pre-hybridized Atlas Human 1.2 cDNA array (2–3 h in MicroHyb solution) and hybridized overnight at 42°C. After hybridization, the filters were washed twice with 2X SSC and 1% SDS solution at 55°C for 20 min followed by a third wash with 0.5X SSC and 1% SDS solution for 15 min. Following the final wash, the filters were exposed to phosphor imaging screens for varying lengths of time to ensure a proper comparison of weakly and strongly expressed genes.

Quantitative analysis of the expressed genes

The phosphor images of hybridized filters were scanned by PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA), and analyzed using Analytical Imaging Station (AIS) software (version 3.0 r.l.3; Imaging Research, St Catharine’s, Ontario, Canada). The intensity of hybridization signals for each spot was determined automatically and corrected for background. Calculated intensities correlate linearly with the concentration of mRNAs present in the total RNA population from both C33 and C81 cells, since the amount of cDNA attached to the membrane was in excess (10 ng) and the background hybridization signals were sufficiently low. For a quantitative difference in gene-expression between arrays (mRNA from C33 and C81), the intensity value of each known gene was normalized in two ways: to the intensity values of the designed housekeeping genes and to the sum of the intensity values of all of the genes. We found no significant difference in the normalized intensity values of all of the genes. Comparison of C33 and C81 RNA population was performed in two separate parallel hybridization experiments. Genes that showed an average induction or reduction of 1.6-fold or higher in both hybridization experiments were considered to be differentially expressed. Correlations and differences in gene expression between C33 and C81 cells were also compared by scatter plot analysis, in which each point represents a particular gene. The location of the point is determined after normalization by the intensity value of the gene in one cell population represented by the x-axis, with its intensity value in the other cell population represented by the y-axis. In each scatter plot, points that lie close to the 45-degree ‘line of identity’ represent genes with similar levels of expression in both LNCaP cells. The perpendicular distance of a point away from the diagonal line represents the degree to which a gene is differentially expressed between C33 and C81 cells of the LNCaP cell model.

Reverse transcription-polymerase chain reaction (RT-PCR)

Oligonucleotide primers were synthesized for genes of interest that showed differential expression in microarray analyses. Total RNA (1 µg) was reverse transcribed using the SUPERSCRIPT™II Rhase H’ Reverse Transcriptase System. Samples were subjected to PCR amplification in a total reaction volume of 50 µl containing 10X PCR buffer (Gibco BRL®), 50 mM MgCl2 (Gibco BRL®), 10 mM dNTP, 5 pmol concentration of each specific primer, and 2.5 units of Taq DNA polymerase (Gibco BRL®). The PCR reaction was carried out in a programmable thermal controller (PTC-100, MJ Research, Inc., Watertown, MA). The reaction mixture was denatured at 94°C for 3 min followed by 30 cycles at 94°C for 45 s, annealing at 60°C for 45 s and 72°C for 1 min. The final elongation was extended for an additional 20 min. For some of the genes, the annealing temperature ranged from 56–60°C. The amplified PCR products were visualized by electrophoresis on a 0.8% agarose gel stained with ethidium bromide to verify size of the amplified product. After nucleotide sequence verification for some of the genes, the PCR products were used to prepare the probes for northern analysis.

Northern blotting

Total RNA (10 µg) was fractionated by gel electrophoresis on 1.0% agarose gel containing 0.66 M formaldehyde and was transferred to nitrocellulose filter via capillary blotting. cDNA probes were labeled with [32P]dCTP using a random primed labeling kit (Pharmacia Biotech, Piscataway, NJ). Pre-hybridization and hybridization of the filter were carried out in a solution of 50% formamide, 5X SSPE, 5X Denhardt’s reagent, 200 µg/ml of sheared salmon sperm DNA, and a minimum of 106 c.p.m./ml of probe at 42°C for 18 h. After hybridization, filters were washed twice with 2X SSC and 0.1% SDS solution at room temperature for 15 min followed by two washes with a solution of 0.2X SSC and 0.1% SDS at 55°C. These filters were exposed to phosphor imaging screens, and scanned with PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Northern blot analysis for the selected genes was performed in duplicates.

Results

Filter hybridization and cDNA array quantification

To address the question of differentially expressed genes during the progression of human prostatic adenocarcinomas, we used a cDNA microarray gene filter (Atlas Human 1.2 cDNA arrays) representing 1176 identified genes. Total RNA from early passage androgen-sensitive (C33), and late passage androgen-independent (C81) LNCaP cells was reverse transcribed, and the generated probes were hybridized to microarray gene filters (Figure 1). On these filters, each spot represents a single known gene separated from the housekeeping genes located at the bottom portion of the filter. The intensity of each spot for the known genes was calculated after normalization of the signals with the housekeeping genes as well as with the background intensity. The distribution profile of the differentially expressed genes is represented by a log transformed scatter plot analysis (Figure 2). The majority of the genes are located close to the ‘line of identity’ (line with a slope of 1) representing no difference in expression, and only a few genes appeared below and above the line of identity, indicating that these genes exhibited ≥2 fold higher expression in either C33 or C81 passage cells. A visual examination of the expression patterns between the filters revealed that some of the genes
Differentially-regulated genes in prostate cancer

Fig. 1. The $^{33}$P-labeled probes from (A) androgen-sensitive C33 and (B) androgen-independent C81 cells of the LNCaP cell model hybridized to microarray gene filters (Human Cancer 1.2: ClonTech). Each cDNA spot corresponds to a single known gene. The six housekeeping genes are located at the bottom. At the upper left and right corners of the gene filter, there is one genomic DNA spot directing the orientation of the filter. The arrow marks indicate differentially-regulated genes confirmed by northern blot analysis (→: up-regulated; ←: down-regulated in C81 stage cells).

showed false positive results. These were subsequently excluded from the study.

**Differential gene expression profile**

A detailed analysis of differentially expressed genes identified in the present study, explaining their localization on the gene filter and the GenBank accession number, is presented in Table I. Out of the 1176 known genes from the Atlas Array 1.2 gene filter, only 42 genes (3.6%) showed differences (1.6-fold or higher) in their level of expression between C33 and C81 cells. The mRNA expression level for 34 genes (2.9%) exhibited up-regulation, while 8 genes (0.7%) displayed down-regulation in C81 cells as compared to C33 cells.

**Confirmation of the gene expression profile by northern blot analysis**

To confirm the quantitative differences in the gene expression profile associated with the progression of the androgen-independent phenotype developed from the androgen-sensitive stage of the LNCaP cell model, northern blot analysis was performed on several selected target genes. These included: c-MYC, c-MYC purine-binding transcription factor (PuF),
macrophage migration inhibitory factor (MIF), macrophage inhibitory cytokine-1 (MIC-1), lactate dehydrogenase-A (LDH-A), guanine nucleotide-binding protein Gi, α-1 subunit (NBP), cyclin dependent kinase-2 (CDK-2), prostate specific membrane antigen (PSM), cyclin H (CCNH), 60S ribosomal protein L10 (RPL10), 60S ribosomal protein L32 (RPL32), and 40S ribosomal protein S16 (RPS16). As shown in Figure 3, data from northern blot analyses of these genes paralleled closely the results obtained by DNA microarrays, indicating these genes indeed have differential expressions in androgen-sensitive and androgen-independent prostate cancer cells.

Discussion

Research in prostate cancer stands to benefit considerably from DNA microarrays, as evidenced by recent studies (16–20,26). Although, certain differentially-regulated genes have been identified, it remains a challenge to prove whether these genes contribute to neoplastic processes or androgen-independency. The molecular events underlying the progression of prostatic adenocarcinomas remain largely unsolved because of the heterogeneity of these tumors. Molecular and biochemical characterization of LNCaP cell model indicates that the cells at various passages mimic tumor progression as seen in clinical patients, indicated by the expression of biomarkers including the elevated secretion of prostate specific antigen (PSA) in androgen depleted conditions (21,23). Other cell models of androgen-independent cells, which were derived under androgen depletion conditions, may represent only a subpopulation of cancer patients, since the expression of biomarkers (such as PSA) in those cells may not be consistent with the clinical phenomenon (27,28). Thus, our LNCaP cell model, to a certain extent, eliminates the possibility of heterogeneity in clinical samples, which is largely responsible for the complexity of this malignancy. Combining the LNCaP cell model with microarray studies has provided an efficient means of identifying genes that are differentially-regulated during tumor progression.

It is clear that in the early stage of prostate tumor progression most of the cells are androgen-sensitive and gradually acquire the phenotypes of androgen-independency. This may be due to the accumulation of genetic and epigenetic changes, leading to the acquisition of androgen-refractiveness. Alternatively, the development and/or progression of the androgen-independent phenotype may arise from a heterogeneous mass of cell populations, i.e. a mixture of androgen-sensitive and androgen-independent cells, in the beginning of cancer. Both these notions are well supported by our recent study with progression of LNCaP cell model (24). Therefore, it is essential to define the sets of genes that facilitate the phenomenon of prostate tumor development and progression.

In this era of functional genomics, microarray technology has provided a great opportunity to analyze the expression profile of thousands of transcripts simultaneously. In this context, to elucidate the transcriptional differences associated with the acquisition of the androgen-independent phenotype of human prostatic adenocarcinomas, we performed the studies using in vitro LNCaP cell model and DNA microarray technology. The microarray gene filters used in the present study contain 1176 known genes, categorized into six groups: (A) oncogenes, tumor suppressors, cell-cycle regulators; (B) transporters, signal transduction; (C) GDP/GTP exchangers and GTPase stimulators/inhibitors, apoptosis; (D) transcription factors, cell signaling and extracellular communication; (E) cell-surface antigens, cell adhesion, receptors; and (F) stress response, cell—cell communication. Further details are available from the ClonTech website, www.clontech.com. It is noteworthy that a large number of genes exhibited up-regulation in C81 cells. Although some of the genes listed in Table I have been confirmed by northern blot analysis, not all of these identified genes are likely to play an important role in tumor progression leading to hormone-refractiveness. Some of these genes are cell cycle regulated, and may be the outcome of the disease. We selected 12 genes, which may potentially play an important role or serve as a useful biomarker for prostate pathogenesis, to confirm their differential expressions by northern blotting. Among these, c-MYC, PuF, LDH-A, MIF, MIC-1, NBP, CDK-2, RPL32, RPL10, RPL16 genes exhibited up-regulation, while PSM and CCNH revealed down-regulation in androgen-independent C81 passage cells.

MYC genes include c-MYC, N-MYC, and L-MYC encoding a family of helix–loop–helix transcription factors containing DNA binding phosphoproteins, which regulate cell growth proliferation and differentiation (29,30). PuF stimulates c-MYC transcription by binding to the promoter of c-MYC gene (31). In this study, the data clearly show that c-MYC and its transcription factor PuF are up-regulated in androgen-independent C81 cells. The results thus correlated well with previous reports of overexpression and amplification of c-MYC gene in prostate cancer (32–34).

Lactate dehydrogenase (LDH) is a tetrameric enzyme comprising two subunits (LDH-A, and LDH-B). LDH is involved in metabolic activities, such as glycolytic pathways. Alterations of the glycolytic pathway, including an enhanced level of LDH-A, are the distinguishing features of cancer cells (35–37). A significantly higher expression of LDH-A and its tyrophosphorylation have been observed in prostatic carcinomas compared with benign prostatic hyperplasia (38,39). In ovarian cancer, tyrophosphorylation of the LDH-A subunit was also observed in both normal and malignant tissues, and the degree of phosphorylation is correlated with the stage of this cancer (40). Interestingly, a higher level of LDH-A is directly correlated with the c-MYC gene up-regulation (37).

It is well established that macrophages are important in orchestrating the chronic inflammatory response that is respons-

Fig. 2. A scatter plot of log transformed data viewing the distribution pattern of differentially regulated genes in androgen-sensitive (C33) and androgen-independent (C81) cells. The solid line indicates the ‘line of identity,’ while the dashed lines indicate >2-fold limits.
Table I. Differentially expressed genes in androgen-sensitive (LNCaP-C33) and androgen-independent (LNCaP-C81) cells of LNCaP cell model identified by microarray analysis

<table>
<thead>
<tr>
<th>Name of the gene</th>
<th>Gene location</th>
<th>Accession number</th>
<th>Fold difference</th>
</tr>
</thead>
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<td>Y15227</td>
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<tr>
<td>MYC proto-oncogene (c-Myc)</td>
<td>A03c</td>
<td>V00568</td>
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<td>C-Myc binding protein MM-1</td>
<td>A03g</td>
<td>D89667</td>
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<td>Cyclin-dependent kinase-2</td>
<td>A03j</td>
<td>M68520</td>
<td>+3.4</td>
</tr>
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<td>Transforming growth factor β-3 (TGF-β3)</td>
<td>A046</td>
<td>J03241</td>
<td>+1.8</td>
</tr>
<tr>
<td>Cell division protein kinase-4 (CDK4)</td>
<td>A04j</td>
<td>M14505</td>
<td>+1.9</td>
</tr>
<tr>
<td>Cyclic B1, G2/mitotic-specific</td>
<td>A05i</td>
<td>M25753</td>
<td>+5.3</td>
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<td>Prohibitin</td>
<td>A06b</td>
<td>S85655</td>
<td>+1.9</td>
</tr>
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<td>N-Myc proto-oncogene</td>
<td>A06c</td>
<td>M13228</td>
<td>+3.1</td>
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<td>Cell cycle progression restoration 2 (CPR2)</td>
<td>A06m</td>
<td>AF011792</td>
<td>+1.9</td>
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<td>L16785</td>
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<td>A11k</td>
<td>X54942</td>
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<td>M80359</td>
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<td>Proto-oncogene RhoA, multidrug resistance protein</td>
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<td>L25080</td>
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<td>STE-20 related kinase DCHT</td>
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<td>High-mobility group protein 2</td>
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<td>X62534</td>
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+ve number indicates up-regulation and the –ve number indicates the down-regulation of the genes in androgen-independent, highly aggressive, late stage cells (LNCaP-C81) of the LNCaP cell model.

*Expression level of these genes was confirmed by northern blot analysis.

Differentially-regulated genes in prostate cancer has been linked to various human diseases such as cervical cancer (7,50), hepatocellular carcinomas (51), heart failure (52), and goiter (53), and stimulates uncontrolled thyroid cell proliferation (54). The up-regulation of cyclin-dependent kinase-2 (CDK-2) in androgen-independent cells reveals its involvement in prostate tumor progression, as the cell cycle is regulated by sequential events of cyclin-dependent kinases (55). In human lung carcinomas, a higher expression of CDK-2 is associated with uncontrolled tumor cell proliferation (56). Simultaneously, three ribosomal proteins (RPL10, RPL32, and RPS16) also showed up-regulation in C81 cells. It is suggested that ribosomal proteins mainly participate in protein synthesis, but additionally they are likely to be involved in the neoplastic transformation of the cells (57,58). Overexpression of several ribosomal proteins including RPS16 has been reported in colon, breast, liver, and pancreatic tumors (59–62). Until this study, analysis of ribosomal protein genes in prostate cancer was very limited.

Although many genes were up-regulated, we also observed down-regulation of several genes in androgen-independent
Fig. 3. Northern blot analysis confirming the expression profile for some of the genes obtained from the microarray gene filter. (A) Up-regulated genes; (B) down-regulated genes in androgen-independent cells of the LNCaP cell model.
cells (Table I). Northern blot analysis further confirmed down-regulation of the selected genes that included the prostate specific membrane antigen (PSM) and cyclin H (CCNH). CCNH is a regulatory component of the cyclin-dependent kinase (CDK)-activating kinase (CAK) that is part of transcriptional factor IIH multiprotein complex. The CAK complex is required for RNA polymerase II transcription, nucleotide excision repair, and phosphorylation of p53 (63,64). Although the functional role of CCNH has not been defined in prostate cancer, its down-regulation can affect regulation of p53 with DNA repair and the basal transcriptional machinery in tumor cells (64). PSM is a membrane-bound glycoprotein highly restricted to prostate (65,66). An alternatively spliced variant, PSM*, is also found in normal prostate. The variant PSM* cDNA is shorter (2387 nucleotides) than PSM (2653 nucleotides) and lacks signal and transmembrane sequences. Prostate tumors and tumor cell lines express predominantly PSM compared to PSM* (67). A higher expression of PSM is seen in hormone-refractory and metastatic conditions and, therefore, has been suggested as a diagnostic marker (68–70). On the other hand, a study on 236 normal individuals and cancer patients by Beckett et al. (71) showed an increased level of PSM in healthy male and female population. Its expression levels increased with age, and the highest level was observed in breast cancer patients. In addition, PSM is not completely restricted to prostatic tissue but also expressed in brain, heart, liver, kidney, lung, and spleen (72). Hence, the use of PSM as a specific biomarker for prostate cancer is not clear. Further investigations are needed in the functional/diagnostic roles of PSM as well as CCNH in prostate cancer progression.

In conclusion, we have identified 34 genes that appear to be up-regulated and eight genes that are down-regulated as LNCaP cells progress in vitro from an androgen-sensitive to androgen-independent phenotype. An association of MIC-1, MIF, and c-MYC genes with prostate tumor progression has already been reported in clinical samples (20,34,45–47). Additional studies will be needed to determine whether the genes identified in this study are key factors in the altered phenotype of the LNCaP cells, and whether these genes can serve as useful biomarkers in androgen-independent prostate cancer cells.

Acknowledgements

This work was supported, in part, by grants from the National Cancer Institute (CA 88184), the Nebraska Health Department/Eppley Cancer Center LB 595 and the Program of Nebraska Research Initiative. We thank Mr Erik Moore and Ms Fen-Fen Lin for their technical assistance, and Ms Kristi L.W.Berger, communications specialist and editor, for editing the manuscript.

References


Differentially-regulated genes in prostate cancer


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Received December 21, 2001; revised February 28, 2002; accepted March 11, 2002