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Gene network and canonical pathway analysis in prostate cancer: a microarray study

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Abbreviations: AR, androgen receptor; BPH, benign prostate hyperplasia; IGF, insulin-like growth factor; PCA, prostate cancer; PSA, prostate specific antigen

Abstract

The molecular mechanism playing a role in the development of prostate cancer (PCA) is not well defined. We decided to determine the changes in gene expression in PCA tissues and to compare them to those in non-cancerous samples. Prostate tissue samples were collected by needle biopsy from 21 PCA and 10 benign prostate hyperplasic (BPH) patients. Total RNA was isolated, cDNA was synthesized, and gene expression levels were determined by microarray method. In the progression to PCA, 738 up-regulated and 515 down-regulated genes were detected in samples. Analysis using Ingenuity Pathway Analysis (IPA) software revealed that 466 network and 423 functions-pathways eligible genes were up-regulated, and 363 network and 342 functions-pathways eligible genes were down-regulated. Up-regulated networks were identified around IL-1β and insulin-like growth factor-1 (IGF-1) genes. The NFKB gene was centered around two up- and down-regulated networks. Up-regulated canonical pathways were assigned and four of them were evaluated in detail: acute phase response, hepatic fibrosis, actin cytoskeleton, and coagulation pathways. Axonal guidance signaling was the most significant down-regulated canonical pathway. Our data provide not only networks between the genes for understanding the biologic properties of PCA but also useful pathway maps for future understanding of disease and the construction of new therapeutic targets.

Keywords: microarray analysis; prostate-specific antigen; prostatic neoplasms

Introduction

Prostate cancer (PCA) is the second most common cancer in men. This tumor develops slowly, and successful screening protocols can help to reduce mortality and morbidity (Müller and Brenner, 2006). Digital rectal examination and serum prostate specific antigen (PSA) determination are the most commonly used screening methods worldwide. PSA has an excellent sensitivity with a low specificity. There is a great interest to find more reliable diagnostic and prognostic markers. The molecular mechanism playing a role in the development of PCA is not known. The heterogeneity of PCA has been recognized for a long time and varies from asymptomatic to a rapidly fatal systemic malignancy (Hughes *et al.,* 2005; Maitland and Collins, 2005). Molecular biological methods are used to determine similarities and differences, and obtain useful information for effective treatment and prognosis (Quinn *et al.,* 2005).

PCA is the leading cause of death in the US and in Europe. It is a heterogeneous disease and the outcome is difficult to predict. Digital rectal examination and serum PSA level measurements are the main tools in the diagnostic protocols.

Molecular biological methods including immunohistochemistry, comparative genomic hybridization (CGH), CGH-array, microarray analysis, and quantitative real-time PCR determinations have been used in the search to determine the molecular mechanism of the developmentof PCA, and to find new diagnostic and prognostic markers (Hughes *et al.,* 2005; Quinn *et al.,* 2005).

Materials and Methods

Patients

Prostate tissue samples were obtained by trans-

rectal ultrasound guided needle biopsy from non-treated 21 PCA (PCA) and 10 benign prostate hyperplasic (BPH) patients from October of 2005 to March of 2006. The selection of patients was based on digital rectal examinations and on serum PSA levels, and was confirmed by histological examination later. Gleason's grades were assigned. The average age was 72.0 $\pm$ 8.8 years vs. 64.3 $\pm$ 12.1 years, respectively. The Ethical Committee of Semmelweis University approved the study and all patients gave informed consent to be involved. Prostate tissue samples were transferred to 1.5 ml Eppendorf tubes containing 0.5 ml of DNA/RNA Stabilization Reagent (Roche, Mannheim, Germany) following the biopsy, and these were kept at -85^{o}C until the RNA isolation.

PSA determination

Pre-biopsy PSA levels were determined by microparticle enzyme immunoassay (MEIA) on an Abbott IMx using a PSA kit (Abbott Park, IL).

RNA isolation

During the RNA isolation tissue samples were placed to lysing matrix tubes containing 0.3 ml of RNA lysis buffer and were treated using a FastPrep Bio101 tissue destroyer centrifuge (Thermo Savant, Holbrook, NY). A Perfect RNA Eukaryotic kit was used (Eppendorf, Hamburg, Germany) for RNA isolation from the samples according to the manufacturer's instructions.

Microarray hybridizations

Applied Biosystems Human Genome Survey Arrays V2.0, covering 29,098 genes, were used to determine the transcriptional profiles of 21 PCA and 10 BPH samples. DIG-UTP labelled cRNA was generated and linearly amplified from 2 μg total RNA using the Chemiluminescent RT-IVT Labeling Kit v 2.0 (Applied Biosystems, Foster City, CA) as described by the protocol. Array hybridization, chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescence Microarray Analyzer following the manufacturer's instructions.

Briefly, each microarray was first pre-hybridized at 55^{o}C for 1 h in hybridization buffer with blocking reagent. Twenty μg DIG-labeled cRNA targets were first fragmented mixed with internal control target and hybridized to the prehybridized microarrays in a volume of 1.5 ml at 55^{o}C for 16 h. After hybridization, the arrays were washed with hybridization wash buffer and chemiluminescence rinse buffer. Enhanced chemiluminescent signals were generated by incubating arrays with alkaline phosphatase conjugated anti-digoxigenin antibody followed by incubation with Chemiluminescence Enhancing Solution and a final addition of Chemiluminescence Substrate. Images were collected for each microarray using the ABI 1700 Chemiluminescent Microarray Analyzer. Images were autogridded and the chemiluminescent signals were quantified, corrected for background and spot and spatially normalized. Obtained data imported into GeneSpring 6.1 software for analysis (GeneSpring 6.1, Silicon Genetics, Redwood City, CA). The fold changes were analyzed by filtering the dataset using P-value $<$ 0.01 and a signal-to-noise ratio $>$ 2 for use in ANOVA statistical analysis. Additional filtering (minimum 2-fold change) was applied to extract the most these gene which were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA). Those genes with known gene symbols (HUGO) and their corresponding expression values were uploaded into the software. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of these genes were algorithmically generated based on their connectivity and assigned a score. The score is a numerical value used to rank networks according to how relevant they are to the genes in the input dataset but may not be an indication of the quality or significance of the network. The score takes into account the number of focus genes in the network and the size of the network to approximate how relevant this network is to the original list of focus genes. The network identified is then presented as a graph indicating the molecular relationships between genes/gene products. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The intensity of the node color indicated the degree of up- or down-regulation. Genes in uncolored notes were not identified as differentially expressed in our experiment and were integrated into the computationally generated networks on the basis of the evidence stored in the IPA knowledge memory indicating a relevance to this network. The node shapes denote enzymes (◊), phosphatases (△), kinases (▽), peptidases (⬭), G-protein coupled receptor (▯), transmembrane receptor (▯), cytokines (□), growth factor (□), ion channel (▯), transporter (⌒), translation factor (⬭), nuclear receptor (▭), transcription factor (⬭) and other (○). Canonical pathways analysis identified the

pathways, from the IPA library of canonical pathways, which were most significant to the input data set. The significance of the association between the data set and the canonical pathway was determined based on two parameters: (1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway and (2) a *P* value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

Quantitative real-time PCR (Q-RT-PCR)

cDNA was synthesized using DNase-I-treated total RNA with a First Stand cDNA synthesis kit for RT-PCR (Roche Diagnostic Corp., Indianapolis, IN) according to the manufacturer's instructions. Quantitative PCR were performed using a LightCycler (Roche) as reported previously (Savli *et al*., 2002) for determination of Fas, IL-1β, P21, and CXCR4 gene expressions. β2-microglobulin gene was used as the internal control. Gene sequences are shown in Table 1. The Relative Quantification REST software package (SPSS, Chicago, IL) was used for the statistical evaluation of the data.

Table 1. List of the primers used for the quantitative RT-PCR.

p21
TGA GCG ATG GAA CTT CGA CT
GAC AGT GAC AGG TCC ACA TGG
β2-Microglobulin
TGA CTT TGT CAC AGC CCA AGA TA
AAT CCA AAT GCG GCA TCT TC
Fas
ACT GTG ACC CTT GCA CCA AAT-
GCC ACC CCA AGT TAG ATC TGG
IL1β
GTGCTGAATGTGGACTCAATCC
TGACAGAGGAGGGTTTCTTAGAAC
CXCR4
CCGCTTCTACCCCAATGACT
GCGAAGAAAGCCAGGATGAG

Results

Differentially expressed genes are shown in two separate tables. The 100 most up-regulated genes (PCA samples compared to BPH samples) are shown in Supplementary Table 1. The 100 most down-regulated genes (BPH samples compared to PCA samples) are shown in Supplementary Table 2. Both sets of results were obtained as two-fold values. Table 2 indicates the gene expression results of four genes (Fas, IL-1β, P21, CXCR4), obtained by Real Time PCR and microarray methods. These results are in a good agreement.

In the progression to PCA 738 up-regulated genes were detected. We investigated interactions using the Ingenuity Pathway Analysis (IPA) software and found 466 network eligible genes and 423 Functions-Pathways eligible genes. Figure 1 shows the most significant three gene networks of over-expressed genes in PCA samples. Top functions of these genes were related to cellular movement, hematological system development, immune response, cell signaling, molecular transport, vitamin and mineral metabolism, cancer, reproductive system, gene expression. These networks identified around IL-1β, IGF-1 and NFKB genes.

Table 3 lists the top ten canonical pathways that are up-regulated. Supplementary Figure 1 shows the canonical pathway of acute phase response signaling, and Supplementary Figure 2 shows the canonical pathway of the coagulation system. Supplementary Figure 3 shows the canonical pathway of hepatic fibrosis/hepatic stellate cell activation in diagrammatic form.

In the progression to PCA 449 down-regulated genes were detected. We investigated interactions using the IPA and found 363 network eligible genes, and 342 Functions-Pathways eligible genes. Figure 2 shows a gene network and down-regulated

Table 2. Summarized real time PCR confirmation results of the four genes.

Genes	Ratios obtained by real-time PCR	Ratios obtained by arrays
IL-1β (interleukin-1 beta precursor)	2418,673 (Up-regulated)	2,541 (Up-regulated)
Chemokine (C-X-C motif) receptor 4	7,754 (Up-regulated)	2,006 (Up-regulated)
Fas (TNF receptor superfamily, member 6)	44,663 (Up-regulated)	2,664 (Up-regulated)
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	221,168 (Down-regulated)	3,153 (Down-regulated)

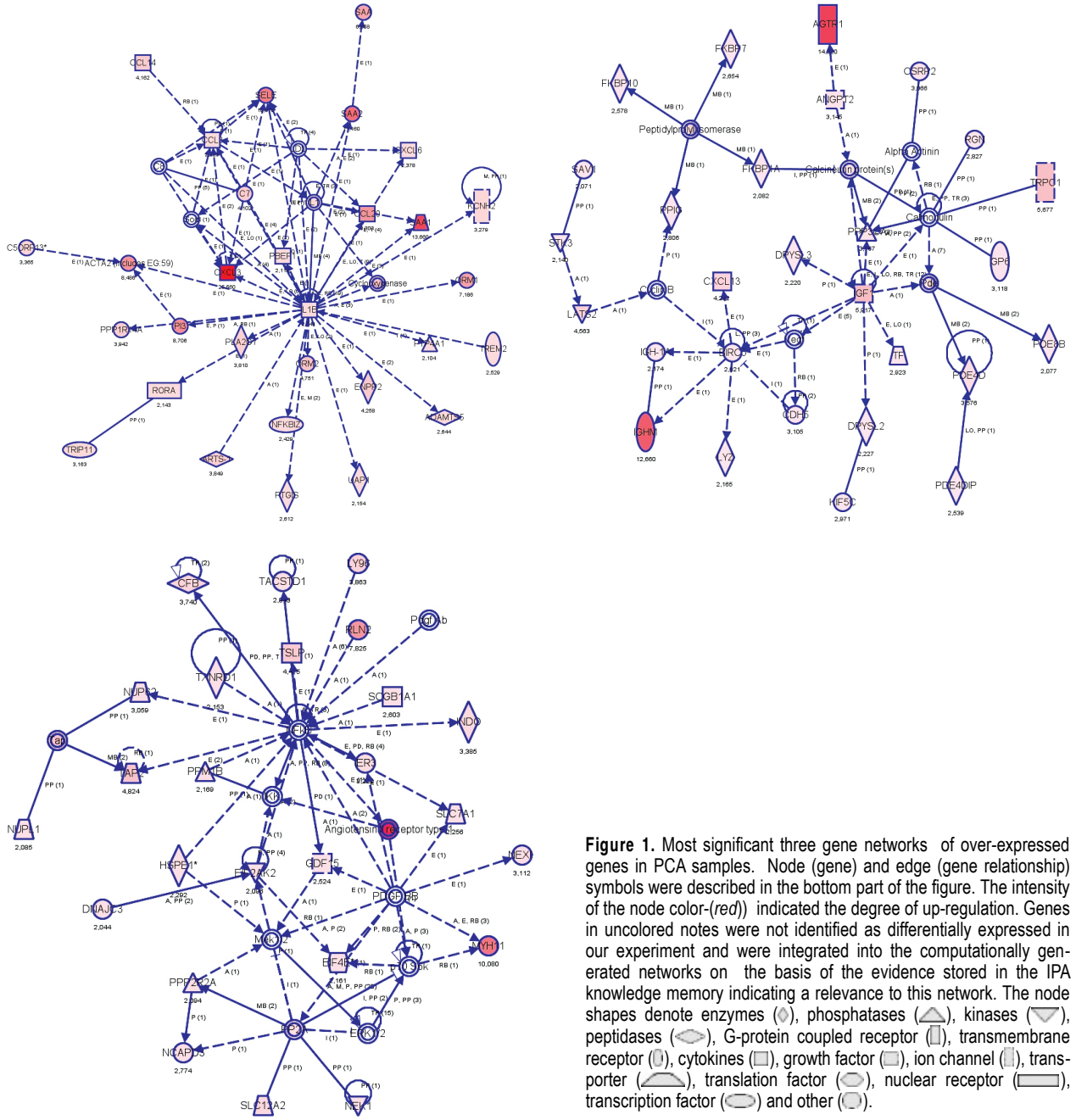

Figure 1. Most significant three gene networks of over-expressed genes in PCA samples. Node (gene) and edge (gene relationship) symbols were described in the bottom part of the figure. The intensity of the node color-(*red*)) indicated the degree of up-regulation. Genes in uncolored notes were not identified as differentially expressed in our experiment and were integrated into the computationally generated networks on the basis of the evidence stored in the IPA knowledge memory indicating a relevance to this network. The node shapes denote enzymes (), phosphatases (), kinases (), peptidases (), G-protein coupled receptor (), transmembrane receptor (), cytokines (), growth factor (), ion channel (), transporter (), translation factor (), nuclear receptor (), transcription factor () and other ().

genes in PCA. The main functions of the genes were related to cellular assembly and organization, connective tissue development and function, and neurological disease, and were identified around the NFKB gene.

Table 4 lists the top ten canonical pathways that are down-regulated. Supplementary Figure 4 shows the canonical pathway of antigen presentation and Supplementary Figure 5 shows the cellular localization of the genes and their functions in the axonal guidance signaling pathway.

Discussion

PCA is a heterogeneous disease and many molecular methods have been used in the search to determine the mechanism of the development of the disease, and to find new diagnostic and prognostic markers (Hughes *et al.,* 2005; Quinn *et al.,* 2005). Our study has demonstrated new genetic networks and biological pathways in both up- and down-regulated gene expression levels. It should be kept in mind that there are certain limits to in

Table 3. List of the genes in most significantly up-regulated top ten canonical pathways.

Pathway	-log (*P*-value)	Ratio	Molecules
Acute phase response signaling	1,33E+01	9,88E-02	TTR, ORM2, C1S, VWF, SAA2, SERPINA3, MAPK12, HRG, ORM1, FOS, RBP7, TF, F8, CFB, IL1B, SAA1, C4B
Hepatic fibrosis/hepatic stellate cell activation	1,26E+01	1,15E-01	FGF2, MYL5, ACTA2 (includes EG:59), EGF, MYH11, MYL9, CXCL3, LY96, EDN1, CCL2, IGF1, IL1B, EDNRA, AGTR1, PDGFRB
NRF2-mediated oxidative stress response	7,54E+00	6,63E-02	CUL3, FOS, PRKCD, ACTA2 (includes EG:59), DNAJB7, DNAJC3, DNAJA2, DNAJC10, ACTG2, ACTC1, TXNRD1, BACH1
Actin cytoskeleton signaling	6,80E+00	5,06E-02	ARHGEF4, FGF2, MYL5, ACTA2 (includes EG:59), EGF, MYH11, WASF1, MYL9, GPR65, PPP1R12B, ACTG2, ACTC1, PDGFRB
Leukocyte extravasation signaling	5,44E+00	5,32E-02	CDH5, MMP3, CXCR4, PRKCD, ACTA2 (includes EG:59), MMP11, ACTG2, MAPK12, MMP23A, ACTC1
Axonal guidance signaling	5,27E+00	3,42E-02	DPYSL2, CXCR4, MYL5, EGF, FZD1, EIF4E, MYL9, EPHA8, IGF1, PRKCD, FZD5, GLI1, PPP3CA, PDGFRB
Calcium signaling	5,12E+00	4,61E-02	MYL9, TPM1, TRPC1, ACTA2 (includes EG:59), MYL5, RCAN2, TPM2, MYH11, ACTC1, PPP3CA
Xenobiotic metabolism signaling	5,10E+00	4,38E-02	CUL3, MAOB, FMO2, CHST7, PPP2R2A, PRKCD, IL1B, UGT2B17, NRIP1, CES1 (includes EG:1066), MAPK12
Coagulation system	4,96E+00	1,43E-01	F8, F5, F9, VWF, F3
Complement system	4,96E+00	1,35E-01	C1S, CFB, CFI, C7, C4B

silico analysis. Since there are many different gene-gene interactions resulting from various cellular/experimental conditions, the edges denoted in the network may not represent the actual causal relationship between genes. Moreover, the probe collection on a chip, which necessarily relies on the coverage and the accuracy of both genomic sequences and clone libraries, presents a serious constraint on its detection power (Liu *et al*., 2007). Furthermore, confounding factors such as low-abundance potential biomarkers could interfere with several groups of high-abundance proteins in human plasma in which a dynamic concentration range of protein component exists (Cho *et al.*, 2005).

Analysis using IPA software revealed 738 up-regulated genes in the progression to PCA. These were 466 network eligible genes and 423 Functions-Pathways eligible genes. Often individual genes were found in multiple categories of functions related to cancer development including cell-to-cell signaling and interaction, cell signaling, cell death, cellular growth and proliferation, andcellular movement.

One important gene network was identified around the IL-1β gene (Figure 1). This gene has been reported to be a potent modulator of cellular differentiation and a suitable target for anticancer drug design in PCA (Albrecht *et al.,* 2004). A group of chemokines (CCL20, CCL3, CXCL3, CXCL6, CCL14) were up-regulated around IL-1β. Chemokines and their ligands are considered to have a significant role in tumor angiogenesis and cancer metastasis. CCL3, CXCL3, and CXCL6 were reported before in PCA (Akashi *et al*., 2006; Engl *et al*., 2006). In particular, CXCL3 were found to be highly up-regulated in our study. Other chemokines (MIP-3, MIP-4, MIP-1A, BCA-1, CC-1, MCP-1, TAFA-5, IP-9, CXCR4) were not identified on this network, but they were up- regulated in our study. The CXCR4 gene was reported as up-regulated in leukemia and PCA before (Savli *et al*., 2002; Engl *et al*., 2006; Ao *et al*., 2007). Akashi *et al.* showed that the expression of androgen receptor (AR) down-regulates the migratory responses of human PCA cells via chemokine and its receptor systems (Akashi *et al*., 2006). AR is an important molecule in the development of a normal prostate and PCA progress (George, 2003). Amplification of the AR gene has been described in more than 30% of tumors to prior to therapy (Nelson *et al.,* 1999). Thus, it was reasonable to suppose that chemo-

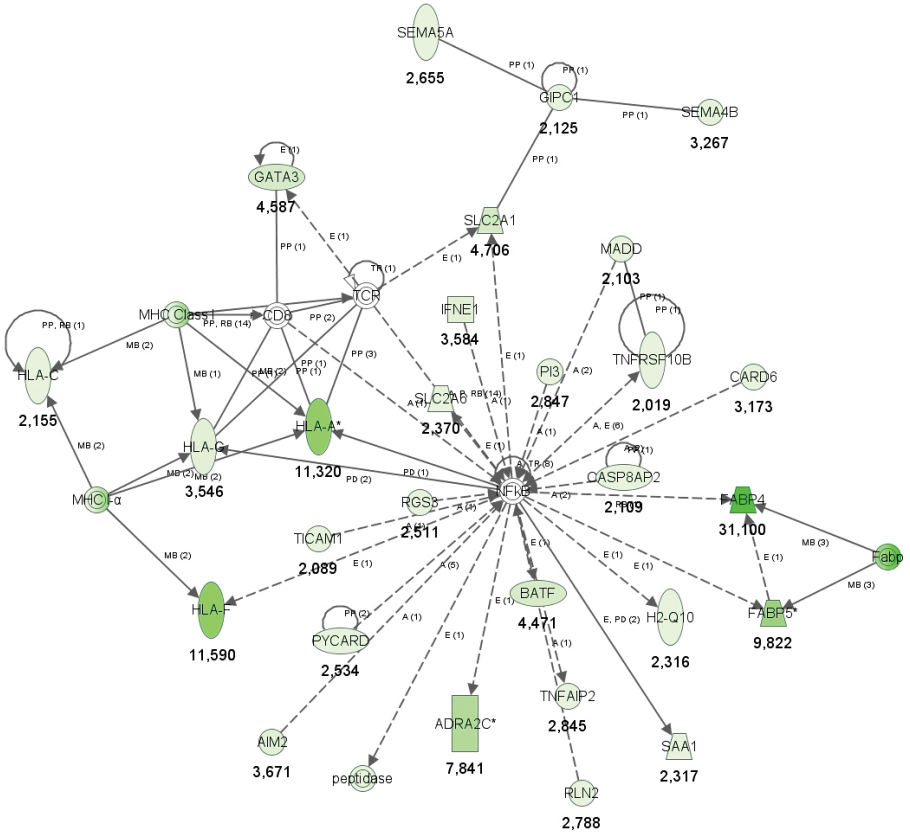

Figure 2. Network contains down-regulated genes and identified around NFKB gene. Top functions of the genes were related to cellular assembly and organization, connective tissue development and function, neurological disease. Node (gene) and edge (gene relationship) symbols were described in the bottom part of the figure. The intensity of the node color (*green*) indicated the degree of down-regulation. Genes in uncolored notes were not identified as differentially expressed in our experiment and were integrated into the computationally generated networks on the basis of the evidence stored in the IPA knowledge memory indicating a relevance to this network. The node shapes denote enzymes (), phosphatases (), kinases (), peptidases (), G-protein coupled receptor (), transmembrane receptor (), cytokines (), growth factor (), ion channel (), transporter (), translation factor (), nuclear receptor (), transcription factor () and other ().

Table 4. List of the genes in most significantly down-regulated top ten canonical pathways.

Pathway	-log (*P*-value)	Ratio	Molecules
Axonal Guidance Signaling	1,16E+01	4,89E-02	PIK3C2B, NGEF, RGS3, SEMA5A, RAC1, KRAS, PLXNA2, GNA14, ROR1 (includes EG:4919), EPHB6, FGFR3, NFAT5, ARPC2, ARHGEF6, GNAO1, ABLIM3, ERBB2, SEMA4B, RTN4R, PPP3CA
Arachidonic Acid Metabolism	1,00E+01	5,69E-02	GRN, LTA4H, PTGES2, AKR1C3, CYP19A1, PLA2G10, GPX2, CYP4Z1, CYP4F3, CYP2J2, ALOX5, CYP2D9
LPS/IL-1 Mediated Inhibition of RXR Function	9,31E+00	6,77E-02	GSTP1, MGMT, ALDH1L1, ABCG1, SULT1A2, MAOB, FABP5, ACSL5, ALAS1, FABP4, HS3ST1, ABCC3, RXRA
ERK/MAPK Signaling	7,94E+00	5,91E-02	PPARG, FGFR3, MYC, PIK3C2B, PPP2CB, ELF3, PLA2G10, PPP2R2B, RAC1, DUSP4, KRAS, ROR1 (includes EG:4919)
Xenobiotic Metabolism Signaling	7,85E+00	5,18E-02	PIK3C2B, GSTP1, UGT1A6, MGMT, ALDH1L1, KRAS, SULT1A2, PPP2CB, MAOB, PPP2R2B, HS3ST1, ABCC3, RXRA
Metabolism of Xenobiotics by Cytochrome P450	7,59E+00	4,69E-02	GSTP1, AKR1C3, UGT1A6, DHRS2 (includes EG:10202), CYP19A1, CYP4Z1, ADH1B, ALDH1L1, CYP2J2, CYP2D9
Antigen Presentation Pathway	6,83E+00	1,54E-01	HLA-DRB4, HLA-A, HLA-G, HLA-DRB5, HLA-F, HLA-C
Eicosanoid Signaling	6,53E+00	8,33E-02	GRN, LTA4H, PTGES2, AKR1C3, PLA2G10, ALOX5, DPEP2
Ephrin Receptor Signaling	6,13E+00	4,81E-02	EPHB6, FGFR3, NGEF, RGS3, ARPC2, GNAO1, RAC1, KRAS, GNA14, ROR1 (includes EG:4919)
Actin Cytoskeleton Signaling	6,07E+00	4,28E-02	FGFR3, PIK3C2B, ACTB, ARPC2, FGF9, ARHGEF6, RAC1, KRAS, ROR1 (includes EG:4919), BDKRB1, TMSB4X

kines and their ligands were highly important in the progression to PCA. Here we present a group of chemokine up-regulation in the most significant gene work of our study.

The SELE gene was a highly up-regulated member of the network around the IL-1β gene. Bhaskar *et al.* (2003) found up-regulated SELE levels in another microarray study on PCA. The role of this gene has been described in metastatic prostate tumor cells as well (Dimitroff *et al.,* 2004). Other adhesion molecules (Selectin P, desmolin, Endothelin1, Endothelin RecA, vimentin, PECAM-1, etc.) were not identified on this network, but they were up-regulated in our study as well.

Three serum amyloid protein coding genes (SAA, SAA1, SAA2) were highly up-regulated in the network around the IL-1β gene. Alterations in the expression of serum amyloid proteins have been linked to many tumors such as osteosarcoma (Kovacevic *et al*., 2008), lung cancer (Dai *et al*., 2007), renal cell carcinoma (Engwegen *et al*., 2007), gastric cancer (Chan *et al*., 2007), and choriocarcinoma (Kovacevic *et al.,* 2007). Serum amyloid protein coding genes are not specific markers for any particular type of tumor. We recommend these genes for monitoring disease activity and response to therapy in patients with PCA.

One network was identified around the insulin-like growth factor 1 (IGF-1) gene (Figure 1). This gene has been linked to PCA before (Abate-Shen and Shen, 2000). Other growth factors (IGFB7, GHBP, URG, and PDF) were not identified on this network, but they were up-regulated in our study.

Another network was identified around the NFKB gene (Figure 1). The angiotensin II receptor type 1 gene was highly up-regulated. This gene could also be a good target for the therapy of prostate cancer (Uemura *et al.,* 2006). Further studies are required to establish the role this gene plays in PCA.

Our study has also demonstrated up-regulated apoptosis related genes (FAS, BIC, PACAP, BFL1) and up-regulated oncogene partners (PET-1, LAF-4, HSP2123-like). FAS is the most conspicuous gene among them. Up-regulated FAS levels were reported in PCA previously (Iacopino *et al.,* 2006). Well-known oncogenes such as Ras (Konishi, 1992), or apoptosis related genes such as BCL2 (McDonnell*,* 1992) were found in PCA progress previously, but we did not confirm them in this study.

Canonical pathway analysis revealed that “acute phase response” was the most significant signaling pathway modulated by the up-regulated genes in PCA (See Supplementary Figure 1). Dysregulation of IL-6-type cytokine signaling was reported to contribute to PCA previously (Heinrich *et al.,* 2003). The genes that we found up-regulated were using the IL1 type cytokine signaling on an extracellular level. Then, interactions were determined among p38 MAPK and c-fos in the nucleus, but it is not easy to determine the pathway that really participated in prostate tumorigenesis since many other pathway members were not detected.

Another significant signaling pathway is hepatic fibrosis/hepatic stellate cell activation (See Supplementary Figure 3). Changes in prostate-specific antigen levels among cirrhotic patients have been reported before (Kubota *et al*., 1999; Akdogan *et al.,* 2002) but further research is needed to establish whether these genes are new molecular markers or not.

The actin cytoskeleton pathway was highly significant. This pathway has been reported many times in PCA before (Moore *et al.,* 2000; Papakonstanti *et al*., 2003; Wells *et al.,* 2005; Marelli *et al.,* 2006). Genes belonging to the coagulation pathway were found to be distinctly up-regulated (See Supplementary Figure 2). Patients with PCA may have life-threatening coagulation complications due to their disease. The pathophysiology of disseminated intravascular coagulopathy in patients with prostate cancer is not completely understood. Investigators reported coagulation disorders as early or late signs in PCA (Bern, 2005; Navarro *et al.,* 2006; Duran and Tannock, 2007; Shirai and Chaudhary, 2007). We found highly up-regulated F8, F5, F9, VWF, and F3 gene accumulation around ca-dependent and ca-phospholipid dependent regions of the coagulation pathway. These regions could be the subject of studies to understand the pathophysiology and plan further therapeutic interventions.

Analysis using IPA software revealed that 515 mapped down-regulated genes were detected in the progression to PCA. These were 363 network eligible genes and 342 Functions-Pathways eligible genes. Often individual genes were found in multiple categories of functions related to cell-to-cell signaling and interaction, cell signaling, immune response, cancer, cellular growth and proliferation, nucleic acid metabolism, cellular assembly and organization, and connective tissue development and function.

One important down-regulated carcinogenic gene network was identified around the NFKB gene (Figure 2). We observed some down-regulated genes, ADRA2C, FABP4, and FABP5, which were not reported in cancer progress before. Moreover, five genes belonging to MHC were strictly down-regulated in this network (H2-Q10, HLA-A, HLA-C, HLA-F, and HLA-G). Tamura *et al.* (2007) observed

down-regulation of HLA-A antigen in microarray analysis of hormone-refractory prostate cancer. We report the down-regulation of four genes (H2-Q10, HLA-C, HLA-F, and HLA-G) here for the first time.

Canonical pathway analysis revealed that "antigen presentation" was an important pathway modulated by the down-regulated genes in PCA. Our findings on MHC genes were related to down-regulation of MHC I-α and MHC II-β class genes (See Supplementary Figure 4).

Axonal guidance signaling was the most significant down-regulated canonical pathway (See Supplementary Figure 5). The FGFR3 gene showed the highest value of under-expression. Fibroblast growth factors (FGFs) and their receptors (FGFRs) have a critical function for the development and homeostasis of the human prostate. Imbalance in expression of these factors is associated with malignancy. We found three members of this family (FGFR3, PIK3C2B, and RAC1) to be down-regulated. RAC1 and FGFR3 were evaluated in previous studies (Kwabi-Addo *et al*., 2001; Gowardhan *et al*., 2005; Chaffer *et al*., 2007; Wu *et al*., 2007), but here we report PIK3C2B gene down-regulation and its relationship to PCA for the first time.Two important gene members of this pathway were down-regulated. K-RAS is not only an oncogene, but also a known target in differentiation therapy of PCA (Benbrahim-Tallaa *et al*., 2005, 2007). ERBB2 overexpression is important in PCA and our finding is in concordance with previous observations (Liu *et al.,* 2001; Calvo *et al*., 2003; Ullén *et al*., 2005). Taken together, two well-known genes, FOS and IL1B, were up-regulated as members of the same pathway as well. Axonal guidance signaling in the progress of PCA needs further investigation.

There are different valuable bioinformatic approaches in the literature to analyze prostate cancer progression. Tomlins *et al*. (2007) profiled prostate cancer progression from benign epithelium to metastatic disease. They identified expression signatures, and analyzed these signatures in the context of a compendium of molecular concepts. Their strategy allowed them to make several insights into disease progression. In the present study we analyzed over one thousand altered genes regarding functions and communications using the IPA tool and detected new relationships.

Our data provide not only new networks between the genes for understanding the biologic properties of PCA but also useful common pathway maps for future understanding of disease and construction of new therapeutic targets. Further combined genomic and proteomic studies are necessary to find out more details of the hierarchy and regulation on DNA-RNA-protein levels.

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