Identification of androgen-regulated genes in human prostate

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Abstract. Androgens are essential for the development of the prostate and prostate cancer. We examined androgen-regulated gene expression in the human prostate. Samples from benign and malignant prostate tissue and samples containing prostate tissue obtained from prostate cancer patients three days after surgical castration were further processed as probes for a GeneChip array. The comparison of gene expression profiles in castrated samples and in benign or malignant prostate tissue samples revealed androgen-regulated genes. We further evaluated the genes which were differentially expressed in benign and malignant prostate samples. The androgen-regulated expression of dual specificity phosphatase 1 (DUSP1) was confirmed in the LNCaP prostate cancer cell line, as the expression of DUSP1 increased with androgen treatment over the course of time. The expression of the genes CRISP3, PCA3, OR51E2, HOXC6, AGR3, AMACR and SLC14A1 was affected by castration in addition to differential expression in the benign and malignant prostate. These sample results require further investigation for the role of AGR3 and SLC14A1 in prostate cancer as these associations have not been reported previously.

Introduction

Androgen action is essential for the development of the prostate and also for the development of prostate cancer. Androgen deprivation therapy is a fundamental treatment for metastasised prostate cancer. The activity of androgens is mediated mainly via the androgen receptor (AR), although other androgen responsive activation mechanisms may exist, especially in hormone-refractory prostate cancer (1). Androgen-regulated gene expression has been investigated in numerous studies using prostate cancer cell lines. However, to the best of our knowledge, only a few studies have used human tissues to evaluate androgen-regulated genes. One study reported gene expression changes following castration using prostatectomy samples after three months of androgen deprivation therapy (combination of anti-androgen and chemical castration) (2). In another study, a comparison was made of the expression profiles of untreated and androgen-independent prostate cancer (3). Mostaghel *et al* (4) studied gene expression changes in human prostate tissue at different time points up to nine months after castration for the treatment of localised prostate cancer. The same group further examined the effect of the 5-α reductase inhibitor dutasteride on prostate gene expression (5). The aim of the present study was to identify androgen-regulated genes in the human prostate. Gene expression analysis was performed on human prostate tissue samples from benign and malignant prostate tissue and from prostate biopsy samples obtained three days after surgical castration.

Materials and methods

Patient samples. Prostate samples were collected from three patients undergoing radical prostatectomy for the treatment of prostate cancer. Prostate biopsy samples were obtained from another three patients three days after surgical castration performed as treatment for prostate cancer. These male individuals had newly diagnosed prostate cancer with no previous hormonal treatments. The biopsies were extracted using a biopsy gun technique and 18 G needles. Six biopsies were obtained and a single prophylactic dose of ciprofloxacin (500 mg) was administered. Written informed consent was obtained from every patient giving tissue samples for the study. The study was approved by the Ethics Council of the Northern Ostrobothnia Hospital District. RNA from the samples was isolated using the QuickPrep Total RNA Extraction kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The RNA from the samples was individually labelled and used for the GeneChip array. Three samples were microdissected and histologically confirmed as benign prostate tissue from radical prostatectomy specimens, and three samples were microdissected and histologically confirmed as Gleason 3+3 prostate cancer tissue

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from radical prostatectomy specimens and three samples were from biopsies taken following surgical castration performed as a therapeutic procedure for prostate cancer.

Cell culture. The prostate cancer cell line LNCaP (CRL-1740) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). LNCaP cell cultures were maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 mM HEPES, 1 mM sodium pyruvate and 2.5 g/l D-glucose or DMEM (Sigma-Aldrich) supplemented with 4,500 mg/l glucose, L-glutamine and 1% penicillin-streptomycin (Invitrogen-Gibco, Carlsbad, CA, USA). The cell cultures were supplemented with 10% foetal bovine serum (FBS) (HyClone, Logan, UT, USA) at 37˚C in a humidified atmosphere of 5% CO_2. FBS was substituted with charcoal-treated FBS in the hormone-induction experiments. The LNCaP cells were plated at $1x10^6$ cells/plate 72 h prior to the experiments. The cells were treated with 10 nM R1881 (PerkinElmer, Boston, MA, USA) or an equal volume of ethanol for 0, 6, 24, or 48 h. Following incubation, the cells were collected, washed with phosphate-buffered saline and used directly for the isolation of RNA.

GeneChip protocol. Experimental procedures for GeneChip were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual following the microarray experiment guidelines. In brief, using 8 μg of total RNA as a template, double-stranded DNA was synthesised using the One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA, USA) and T7-(dT)24 primers. The DNA was purified using a GeneChip Sample Cleanup Module (Qiagen, Venlo, The Netherlands). *In vitro* transcription was performed to produce biotin-labeled cRNA using an *in vitro* transcription labeling kit (Affymetrix), according to the manufacturer's instructions. Biotinylated cRNA was cleaned with a GeneChip Sample Cleanup Module (Qiagen), fragmented to 35-200 nucleotides and hybridised to Affymetrix Human Genome U133 Plus 2 arrays that contained ~55,000 human transcripts. After being washed, the array was stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA). The staining signal was amplified using biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA, USA) and a second staining was performed with streptavidin-phycoerythrin before the array was scanned on a GeneChip Scanner 3000. The expression data were analysed using Affymetrix GeneChip Operating System Software. Signal intensities of all probe sets were scaled to the target value of 500.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from LNCaP cells for quantitative RT-PCR measurements was isolated with TRIzol reagent (Invitrogen-Gibco) according to the manufacturer's instructions. Using the First-Strand cDNA synthesis kit (Amersham Biosciences) the first-strand cDNA was synthesised with 1 μg of RNA and pd(N)6 random hex deoxynucleotides according to the manufacturer's instructions. The mRNA levels for LNCaP cells were measured by quantitative RT-PCR analysis (ABI 7700, Applied Biosystems, Foster City, CA, USA) as described previously (6). The forward and reverse primers for for the detection of dual specificity phosphatase 1 (DUSP1) mRNA were 5'-TCCTTCTTCGCTTTCAACGC-3' and 5'-ACGATGGTGCTGAAGCGC-3', respectively. Amplicons were detected using the fluorogenic probe 5'-FAM-CACA TCGCCGGCTCTGTCAACG-TAMRA-3'. The primers and probe for the 18 S amplicon were 5'-TGGTTGCAAAGCTGA AACTTAAAG-3' (forward), 5'-AGTCAAATTAAGCCGCA GGC-3' (reverse) and 5'-VIC-CCTGGTGGTGCCCTTCCG TCA-TAMRA-3', respectively.

Analysis of gene expression profiles. The expression profiles from the GeneChip array were analysed using Chipster software (http://chipster.csc.fi). The GeneChips were normalised using the robust multiarray average (RMA) method and gene expression intensity estimates were received in log2-transformed values. The pathways involved were identified using Chipster software utilising data from ConsensusPathDB (7). The data discussed in this study have been deposited in the NCBI's Gene Expression Omnibus (8) and are accessible through GEO Series accession number GSE32982 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32982).

Statistical analysis. Statistical analysis of the levels of DUSP1 mRNA in LNCaP cells was performed using SPSS version 15.0 (SPSS, Chicago, IL, USA). The Student's t-test was used for comparison between the two groups. Values were presented as the means ± SD.

Results

Genes differentially expressed in prostate cancer and benign prostate tissue, and genes differentially expressed in benign prostate tissue or prostate cancer and castrated prostate cancer are listed in Table I. These genes contain several well-known androgen-regulated genes and prostate cancer-associated genes, including MSMB, SORD, CRISP3 and PCGEM1. The consensus pathways involved between benign and malignant tissue and between benign tissue and castrated prostate cancer are shown in Table II.

The androgen-regulated expression of DUSP1 in LNCaP prostate cancer cell lines was also evaluated (Fig. 1). The treatment of LNCaP cells with synthetic androgen R1881 led to an increased expression of DUSP1 mRNA, with a peak of 2.6-fold expression following 48 h of treatment compared with androgen-depleted conditions.

Discussion

To identify androgen-regulated genes in the human prostate, we used human prostate tissue mRNAs in a GeneChip array. The main limitation of widely used human prostate cancer cell lines such as LNCaP, PC-3 and DU-145 is that they do not represent the prostate *per se*, but are isolated from a prostate cancer metastasis. We evaluated the gene expression profiles from microdissected tissue samples from freshly prepared radical prostatectomy samples and from transrectal prostate biopsies obtained three days after surgical castration performed as a treatment for prostate cancer.

Certain known androgen-regulated genes were identified, including MSMB (9) and SORD (10). Furthermore, a number

Table I. Differentially expressed genes in benign prostate tissue samples, prostate cancer samples and samples obtained following surgical castration. [a]

Symbol	Description	P-value	Fold overexpression	
			Cancer vs. benign	Benign/cancer vs. castrated
CRISP3	Cysteine-rich secretory protein 3	<0.001	5.78	-3.08
FOS	FBJ murine osteosarcoma viral oncogene homologue	<0.001		4.76
PCGEM1	Prostate-specific transcript 1 (non-protein coding)	<0.001		4.42
MYBPC1	Myosin binding protein C, slow type	<0.001		4.30
PCA3	Prostate cancer antigen 3 (non-protein coding)	<0.001	4.18	3.74
OR51E2	Olfactory receptor, family 51, subfamily E, member 2	<0.001	4.01	3.29
ANPEP	Alanyl (membrane) aminopeptidase	<0.001		3.84
DPP4	Dipeptidyl-peptidase 4	0.005		3.79
GCNT2	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme (I blood group)	0.001		3.73
HOXC6	Homeobox C6	0.003	3.69	-2.62
AGR3	Anterior gradient homologue 3 (*Xenopus laevis*)	0.003	3.63	3.00
RGS1	Regulator of G-protein signalling 1	0.002		3.55
NR4A2	Nuclear receptor subfamily 4, group A, member 2	0.002		3.50
DUSP1	Dual specificity phosphatase 1	0.011		3.48
AMACR	α-methylacyl-CoA racemase	0.008	3.40	3.00
LOC728606	Hypothetical LOC728606	0.015		3.36
DLX1	Distal-less homeobox 1	0.011	3.30	
VEGFA	Vascular endothelial growth factor A	0.004		3.30
MSMB	Microseminoprotein, β-	0.006		3.21
TDO2	Tryptophan 2,3-dioxygenase	0.025		3.19
NCAPD3	Non-SMC condensin II complex, subunit D3	0.008		3.14
EGR1	Early growth response 1	0.027		3.14
CD38	CD38 molecule	0.008		3.13
C15orf48	Chromosome 15 open reading frame 48	0.022	3.11	
RASD1	RAS, dexamethasone-induced 1	0.030		3.09
AGR2	Anterior gradient homologue 2 (*Xenopus laevis*)	0.023	3.07	
SORD	Sorbitol dehydrogenase	0.012		3.02
C12orf56	Chromosome 12 open reading frame 56	0.037		3.01
SFTPA2	Surfactant protein A2	0.037		2.99
ST6GALNAC1	ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminide α-2,6-sialyltransferase 1	0.038		2.98
ACADL	Acyl-CoA dehydrogenase, long chain	0.013		2.98
EGR3	Early growth response 3	0.044		2.94
ERG	V-ets erythroblastosis virus E26 oncogene homologue (avian)	0.045	2.90	
FOSB	FBJ murine osteosarcoma viral oncogene homologue B	0.016		2.90
SCD	Stearoyl-CoA desaturase (δ-9-desaturase)	0.024		2.80
GNMT	Glycine N-methyltransferase	0.028		2.76
CCK	Cholecystokinin	0.028		2.75
PEBP4	Phosphatidylethanolamine-binding protein 4	0.031		2.72
ATF3	Activating transcription factor 3	0.031		2.71
HLA-DQA1	Major histocompatibility complex, class II, DQ α 1	0.032		2.70

Table I. Continued.

Symbol	Description	P-value	Fold overexpression	
			Cancer vs. benign	Benign/cancer vs. castrated
SELE	Selectin E	0.035		2.66
PENK	Proenkephalin	0.043		2.61
CD177	CD177 molecule	0.048		2.58
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1 (non-protein coding)	0.032		-2.69
ASPN	Asporin	0.029		-2.74
PSPH	Phosphoserine phosphatase	0.027		-2.77
SLC14A1	Solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	0.048	-2.88	-3.05
HBB	Haemoglobin, β	0.016		-2.89
SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin)	0.045	-2.91	
KRT14	Keratin 14	0.032	-2.99	
NEFH	Neurofilament, heavy polypeptide	0.022	-3.10	
TMEM45B	Transmembrane protein 45B	0.007		-3.16
GPM6A	Glycoprotein M6A	0.027		-3.17
RLN1	Relaxin 1	0.016	-3.21	
GREM1	Gremlin 1	0.004		-3.29
MME	Membrane metallo-endopeptidase	0.004	-3.57	
CXCL13	Chemokine (C-X-C motif) ligand 13	0.003	-3.67	
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5	0.001	-3.92	
WIF1	WNT inhibitory factor 1	<0.001	-4.14	

[a]Gene symbols and gene descriptions are presented. P-values for differential expression were produced using Chipster software. Only genes with P-values <0.05 are presented. Negative values in the fold overexpression column indicate that the gene was underexpressed in cancer compared with benign samples or underexpressed in benign or cancer samples compared with castrated samples.

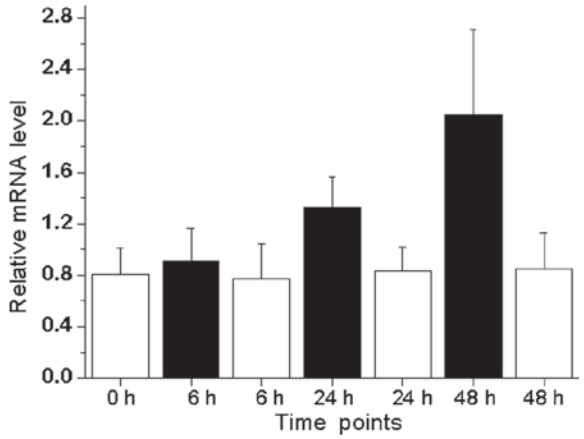

Figure 1. Response of DUSP1 mRNA in LNCaP cells following treatment with 10 nM synthetic androgen R1881 (solid bars). Statistically significant induction of DUSP1 mRNA was observed after 24 and 48 h compared with the control after 24 and 48 h with P=0.04 and P<0.001, respectively. The values are the means ± standard deviations of four individual samples. DUSP1, dual specificity phosphatase 1.

of previously identified prostate cancer-associated genes, such as CRISP3 (11), PCGEM1 (12), PCA3 (13) and OR51E2 (also known as PSGR) (14) were also differentially expressed.

Cholesterol biosynthesis has several correlations with prostate cancer. Low serum cholesterol levels have been correlated with a lower risk of high-grade prostate cancer (15). Furthermore, the use of cholesterol-lowering drugs, such as statins, is associated with a lower risk of advanced prostate cancer (16). Increased cholesterol synthesis may serve as a precursor for intratumoural androgen synthesis in castration-resistant prostate cancer (17).

Focal adhesion pathways are involved in gene-expression changes between benign and malignant prostate tissue. Cell adhesion is a significant process in cancer (18). The steroid biosynthesis pathway was found to be active between the castrated and benign tissue, as expected.

DUSP1 (also known as mitogen-activated protein kinase phosphatase 1, MKP1) is androgen-regulated in the rat prostate (19). DUSP1 expression was detected in prostate cancer with a decreased expression in poorly differentiated carcinomas. Moreover, DUSP1 expression was downregulated in androgen-depleted clinical prostate cancer samples and the expression of DUSP1 was inversely correlated with apoptosis (20). In prostate cancer specimens, the expression of DUSP1 was low in hormone-refractory prostate cancer, whereas it was high in benign prostatic hyperplasia samples and in non-hormone-treated prostate cancer (21). The results of our study confirm those of previous results as DUSP1 expression was

Table II. Pathways potentially active in the transition from benign to malignant prostate tissue and during surgical castration based on gene-expression differences.[a]

Pathway	Database	P-value	
		Benign tissue vs. cancer	Castrated tissue vs. benign tissue
AP-1_transcription_factor_network	PID	0.007	<0.001
Beta1_integrin_cell_surface_interactions	PID	<0.001	
Bevacizumab_Pathway	SMPDB	0.006	
Cholesterol_biosynthesis	Wikipathways		<0.001
Cholesterol_biosynthesis	Reactome		0.002
cholesterol_biosynthesis_I	HumanCyc		0.003
cholesterol_biosynthesis_II_(via_24,25-dihydrolanosterol)	HumanCyc		0.003
cholesterol_biosynthesis_III_(via_desmosterol)	HumanCyc		0.003
Collagen_adhesion_via_alpha_2_beta_1_glycoprotein	Reactome	0.001	<0.001
ECM-receptor_interaction_-_Homo_sapiens_(human)	KEGG	<0.001	
Fatty_Acyl-CoA_Biosynthesis	Reactome		0.007
Focal_Adhesion	Wikipathways	0.004	
Focal_adhesion_-_Homo_sapiens_(human)	KEGG	0.003	
GPCR_signalling-cholera_toxin	INOH	0.004	0.003
GPCR_signalling-G_alpha_i	INOH		0.006
GPCR_signalling-pertussis_toxin	INOH		0.006
HIF-1-alpha_transcription_factor_network	PID		0.008
Immunoregulatory_interactions_between_a_Lymphoid_and_a_non-Lymphoid_cell	Reactome	<0.001	<0.001
Integrin	INOH	0.006	
Integrins_in_angiogenesis	PID	0.004	
Ketogenesis	HumanCyc		0.006
Neurophilin_interactions_with_VEGF_and_VEGFR	Reactome	0.005	
Platelet_degranulation_	Reactome	0.006	
Prostaglandin_Synthesis_and_Regulation	Wikipathways	0.007	
Protein_digestion_and_absorption_-_Homo_sapiens_(human)	KEGG	0.002	
Response_to_elevated_platelet_cytosolic_Ca^{2+}	Reactome	0.007	
Signalling_by_PDGF	Reactome	<0.001	
Signalling_by_VEGF	Reactome	0.008	
Smooth_Muscle_Contraction	Reactome	0.003	
Steroid_Biosynthesis	SMPDB		<0.001
Steroid_biosynthesis_-_Homo_sapiens_(human)	KEGG		0.002
Superpathway_of_cholesterol_biosynthesis	HumanCyc		<0.001
Syndecan-1-mediated_signalling_events	PID	0.001	
Valine_degradation_I	HumanCyc		0.009
Vatalanib_Pathway	SMPDB	0.006	
VEGF_ligand-receptor_interactions	Reactome	0.008	

[a]The active pathways identified and the respective P-values were produced using Chipster software. Databases used for the identification of the pathways are presented. PID, pathway interaction database; SMPDB, small molecule pathway database; KEGG, Kyoto encyclopedia of genes and genomes; INOH, integrating network objects with hierarchies.

3.48 times lower in castrated samples compared with benign or malignant prostate samples (Table I). We also provide data for the androgen-mediated induction of DUSP1 in androgen-sensitive LNCaP prostate cancer cell lines (Fig. 1). Taken together, it appears that DUSP1 is not important in hormone-refractory prostate cancer, as increased apoptosis detected in

androgen-depleted prostate cancer by Magi-Galluzzi *et al* (20) may be explained by the low expression of DUSP1 under androgen-depleted conditions and high apoptotic indices under the same conditions in hormone-sensitive prostate cancer. The low expression of DUSP1 in hormone-refractory prostate cancer (21) may be explained by the androgen-dependent expression of DUSP1 shown in our study and previously by Leav *et al* (19), as castration is an ongoing process in hormone-refractory prostate cancer.

Of note are the genes that are differentially expressed in benign tissue and cancer, which are also overexpressed or underexpressed following castration, meaning that the gene is regulated by androgens and potentially involved in prostate carcinogenesis. These genes are CRISP3, PCA3, OR51E2, HOXC6, AGR3, AMACR and SLC14A1 (Table I). To the best of our knowledge, the association between prostate cancer and AGR3 or SLC14A1 has yet to be established. A high level of the immunoexpression of AGR3 has been linked with prolonged survival in ovarian cancer (22) and AGR3 expression has previously been detected in breast tumours (23). SLC14A1 was recently described as a urinary bladder cancer susceptibility gene (24).

Certain well-known androgen-regulated genes, such as kallikreins (25,26), are not shown in Table I. This is due to the variation in gene expression levels in different samples leading to increased P-values. Thus genes, such as kallikreins, did not exceed the given threshold level of $P<0.05$ for genes presented in Table I (data not shown). The number of samples analysed in our study was limited (three samples per group) and the biopsies obtained from castrated patients were random biopsies, thus these samples may represent cancer or benign tissue, or both. Despite these limitations, our data may be valuable for further studies in prostate cancer.

In conclusion, we have described the identification of androgen-regulated genes in the human prostate, some of which are potential new diagnostic or therapeutic targets in prostate cancer. Particular attention should be paid to AGR3 and SLC14A1 for their roles in prostate cancer. Furthermore, these gene expression profiles may be useful in sophisticated gene expression analyses utilising expression profiles from several different sources.

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