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Decrease and Gain of Gene Expression Are Equally Discriminatory Markers for Prostate Carcinoma

A Gene Expression Analysis on Total and Microdissected Prostate Tissue

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Information on over- and underexpressed genes in prostate cancer in comparison to adjacent normal tissue was sought by DNA microarray analysis. Approximately 12,600 mRNA sequences were analyzed from a total of 26 tissue samples (17 untreated prostate cancers, 9 normal adjacent to prostate cancer tissues) obtained by prostatectomy. Hierarchical clustering was performed. Expression levels of 63 genes were found significantly (at least 2.5-fold) increased, whereas expression of 153 genes was decreased (at least 2.5-fold) in prostate cancer *versus* adjacent normal tissue. In addition to previously described genes such as hepsin, overexpression of several genes was found that has not drawn attention before, such as the genes encoding the specific granule protein (*SGP28*), α-methylacyl-CoA racemase, low density lipoprotein (LDL)-phospholipase A2, and the anti-apoptotic gene *PYCR1*. The radiosensitivity gene *ATDC* and the genes encoding the DNA-binding protein inhibitor *ID1* and the phospholipase inhibitor uteroglobin were significantly down-regulated in the cancer samples. DNA microarray data for eight genes were confirmed quantitatively in five normal and five cancer tissues by real-time reverse transcriptase-polymerase chain reaction with a high correlation between the two methods. Laser capture microdissection of epithelial and stromal compartments from cancer and histological normal specimens followed by an amplification protocol for low levels of RNA (<0.1 μg) allowed us to distinguish between gene expression profiles characteristic of epithelial cells and those typical of stroma. Most of the genes identified in the non-microdissected tumor material as up-regulated were indeed overexpressed in cancerous epithelium rather than in the stromal compartment. We conclude that development of prostate cancer is associated with down-regulation as well as up-regulation of genes that show complex differential regulation in epithelia and stroma. Some of the gene expression alterations identified in this study may prove useful in the development of novel diagnostic and therapeutic strategies. *(Am J Pathol 2002, 160:2169–2180)*

Adenocarcinoma of the prostate is the most frequent cancer in males in western countries. Of major public health importance is the development of reliable discriminating strategies for early detection of neoplastic disease.[1]

Levels of serum prostate-specific antigen, a serine protease, frequently serve as a diagnostic marker for prostate cancer, although elevated concentrations can also be found in benign prostatic hyperplasia and acute and chronic inflammation.[2] Histopathological diagnosis of prostate carcinoma is still regarded as the decisive standard in clinical practice. Tumors are graded as proposed by Gleason.[3] This grading system relies on histological patterns of glandular differentiation. Patient group survival can be determined quite reliably when grading is used in combination with tumor stage.[4] Morphologically similar tumor types can show different biological behavior, however.

Precancerous lesions are referred to as prostatic intraepithelial neoplasia. Although prostatic intraepithelial

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neoplasias constitute highly predictive markers for adenocarcinoma, prostatic intraepithelial neoplasias are based on diagnostic criteria that are subject to a certain degree of subjectivity as is diagnosis of different degrees of epithelial dysplasias in general.[5] Postatrophic hyperplasia, which can be characterized by small densely packed glands with an increased nuclear/cytoplasm ratio, sometimes can be difficult to distinguish from prostatic adenocarcinoma.[6] Postatrophic hyperplasia or proliferative inflammatory atrophy have been implicated in prostatic carcinogenesis.[6,7]

Gene expression profiling is now being considered as an objective supplementary approach to the histopathological work-up of precancerous or cancerous lesions of the prostate. Using high-density microarrays with a large collection of cDNAs or gene-specific oligonucleotides one can identify marker genes or clusters of genes the altered expression of which is characteristic of specific stages of tumor disease.[8–11]

Laser-assisted microdissection of atypical glandular structures and subsequent analysis of multiple genes with DNA arrays or of single marker genes by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) is a powerful refinement to gene expression profiling protocols and is likely to enhance the diagnostic value of gene expression data.[12] This approach excludes contribution of RNA from fibromuscular tissue and tumor-infiltrating mononuclear cells to the gene expression profile. An additional advantage is that it may be potentially applicable to prostate biopsies obtained in preoperative diagnostic procedures.

In this study gene expression profiles were generated from adenocarcinoma of the prostate and from adjacent normal tissue resected from patients not previously treated by chemotherapy or radiotherapy. Profiling of microdissected glands and stroma, both normal and cancerous, was also performed. The results show that carcinoma can be differentiated from histological nontumorous prostate by both significant increases as well as decreases in expression of specific genes, some of which have not been identified previously in conjunction with gene expression patterns in prostate cancer.

An association between marker gene expression and carcinoma may be used to enhance the diagnostic value of the pattern-oriented histological grading system that is currently in use. Analysis of gene expression profiles can also reveal metabolic or signal transduction pathways that might be targeted by new therapeutic strategies.

Materials and Methods

Prostate Tissue

Prostate cancer tissue samples were obtained from patients who had undergone radical prostatectomy for prostate cancer. None of the patients included in this study had received preoperative hormonal therapy, chemotherapy, or radiation therapy. Seventeen primary cancers and 9 normal adjacent to cancer tissues were examined. Collection of tissue and use for this study were approved according to standard guidelines by the ethics committee of the Medical Faculty of the University of Heidelberg. Table 1 shows ages, pre- and postoperative serum prostate-specific antigen concentrations, Gleason scores, and staging of all patients from whom prostate tissue was obtained for this study. After radical prostatectomy, tissues were flash-frozen in liquid nitrogen and stored at −80°C. Seven-μm sections were cut with a standard cryostat and stained with hematoxylin and eosin to identify tumor-free (N1 to N9) and tumorous tissue parts (T1 to T17); cancerous tissue was graded according to the Gleason scoring system[3] by a pathologist. The nonmalignant samples contained predominantly epithelial cells and relatively low amounts of fibromuscular stroma cells; nevertheless the ratio of epithelial cells to stroma was higher in cancer than in tumor-free parts of prostate cancers.

RNA Isolation and Oligonucleotide Array Hybridization

Total RNA was extracted by the method of Chomczynski and Sacchi.[13] RNA quality was monitored by agarose gel electrophoresis; 20 μg of total RNA were reverse-transcribed using Superscript II reverse transcriptase (Life Technologies Inc., Gaithersburg, MD), then converted into double-stranded cDNA, and biotin-labeled during *in vitro* transcription from the T7 promoter using the ENZO RNA Transcript labeling kit; all reactions were performed essentially according to the Affymetrix protocol (Affymetrix, Sunnydale, CA). Each sample was tested for RNA integrity by hybridization to Affymetrix Test2 Chips. Only cRNA samples that passed this test were used on Human Genome U95A chips (HG-U95A, ~12,600 sequences; the list of genes is available at *www.affymetrix.com*). The default average intensity of all mRNAs on a chip was uniformly set at 1000, signals below a signal intensity of 200 were disregarded. Under such conditions, the reproducibility of two identical samples (T7 and T7R) from one tumor resulted in a correlation coefficient of $r = 0.98$ (not shown). Usually, ~60% of the sequences on a HG-U95A chip gave a present call.

Quantitative Real-Time RT-PCR for Confirmation of Microarray Data

RT-PCR products from five cases of the normal group (N1, N3, N4, N5, N6) and five cases of the cancer group (T10, T14, T15, T16, T17) were used to confirm the microarray data by quantitative real-time RT-PCR. The PCR reactions were performed in the LightCycler apparatus using the LC-FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany).

Two μg of the same total RNA used for microarray assay were used for the first-strand cDNA synthesis with Superscript II reverse transcriptase and oligo $d(T)_{12–18}$ primer according to the manufacturer's protocol (Life Technologies).

Table 1. Clinicopathological Information

Case	Age, years	PSA* [ng/ml] preoperative	PSA* [ng/ml] postoperative	Gleason†	Stage‡
Normal group					
N1	52	34.0	n.a.		pT3c, pN0, pMx
N2	65	12.4	0.3		pT2b, pN0, pMx
N3	69	16.0	0.2		pT3a, pN0, pMx
N4	59	1.0	<0.1		pT2b, pN0, pMx
N5	70	58.0	<0.1		pT2b, pN0, pMx
N6	52	3.6	0.2		pT2a, pNx, pMx
N7	44	n.a.	n.a.		pT2a, pN0, pMx
N8	65	8.1	0.9		pT2b, pNx, pMx
N9	62	4.4	<0.1		pT3b, pN0, pMx
Cancer group					
T1	66	10.7	<0.1	3 + 3	pT2a, pN0, pMx
T2	63	6.4	0.2	3 + 3	pT3, pN0, pMx
T3	59	27.0	<0.1	4 + 5	pT3b, pN0, pMx
T4	77	59.0	16.1	3 + 4	T3, Nx, M1
T5	62	6.9	<0.1	3 + 4	pT3a, pN0, pMx
T6	69	16.0	0.2	3 + 4	pT3a, pN0, pMx
T7	68	13.3	0.1	3 + 3	pT3a, pN0, pMx
T8	62	59.0	2.2	3 + 4	pT3, pN0, pMx
T9	63	6.1	<0.1	3 + 4	pT2b, pNx, pMx
T10	77	12.8	<0.1	3 + 4	pT3a, pN0, pMx
T11	72	1.5	0.2	4 + 3	pT3b, pN1, pMx
T12	71	48.6	0.7	3 + 4	pT3b, pN0, pMx
T13	65	8.1	0.9	3 + 3	pT2b, pNx, pMx
T14	63	82.0	0.2	5 + 4	pT3b, pN1, pMx
T15	61	34.4	<0.1	4 + 3	pT4, pN0, pMx
T16	62	4.4	<0.1	3 + 4	pT3b, pN0, pMx
T17	64	37.9	0.1	5 + 4	pT4, pN0, pMx

*PSA, prostate specific antigen.
†Cancer tissue was evaluated according to the histological Gleason scoring system.
‡Clinical and histopathological (p) staging after prostatectomy according to the TNM classification.
n.a., not available.

The primer sequences used in this study are given in Table 2. We used eight genes (five genes found to be increased in microarray assay and three that were decreased) for confirmation by the LightCycler. After optimizing of all PCR reactions at the same annealing temperature of 60°C, thermocycling for each reaction was performed in a final volume of 20 μl containing 2 μl of cDNA sample, 4 mmol/L $MgCl_2$, 0.5 μmol/L of each primer, and 2 μl of LC-FastStart DNA Master SYBR Green I. After 480 seconds of initial denaturation at 95°C, the cycling conditions of 45 cycles for each gene consisted of denaturation at 95°C for 15 seconds, annealing at 60°C (for all genes) for 5 seconds, elongation at 72°C for 10 seconds, and a short temperature increase to 82°C for 3 seconds (for fluorescence measurement). For preparing the standard curve, we used *GAPDH* as the reference gene because it showed similar expression levels in normal and cancer samples (data from microarray assay) and it was amplified with an efficiency similar to seven of eight genes that were confirmed by RT-PCR. Serial dilu-

Table 2. Sequences of Primers Used in This Study

Gene	Sense	Anti-Sense	Amplicon, bp
LightCycler			
Alpha-methylacyl-CoA racemase	5′-AATGTAGAAAATGAGGAAATGCC-3′	5′-AGTTTGGAATGTGCTTAGAGGG-3′	125
LDL-phospholipase A2	5′-CATGGGTTTATAGTTGCTGCTG-3′	5′-GCTTGGGAACATTCTTTTGC-3′	188
Hepsin	5′-CCAAGGACACCCTCCCTC-3′	5′-AAGAGCATCCCATCATCAGG-3′	152
Pyrroline 5-carboxylase reductase 1	5′-CCTGAGAGCAAAGGTCAAGG-3′	5′-GACAGAACTGATAGCACCCTCC-3′	295
DNA-binding protein inhibitor ID1	5′-ATTTCTTCTCGTTTTCACAGGC-3′	5′-TCGGTCTTGTTCTCCCTCAG-3′	175
ATDC	5′-GTGCTCTCTCTCGTCCTACCTATC-3′	5′-AATATCTTGGCTAAGGTCATCCTG-3′	193
Uteroglobin	5′-TCATAACTGGAGGGTGTGTCC-3′	5′-ACCCATGAAAACTCGCTGTC-3′	136
GAPDH (M33197)	5′-CAACTACATGGTTTACATGTTC-3′	5′-GCCAGTGGACTCCACGAC-3′	181
ERG (M21535)	5′-AAGGTGGGACTGAGGATGTG-3′	5′-CAAACAAAGAAAGAGATGCGC-3′	290
TaqMan			
GRO-2 oncogene	5′-CGCAGCAGGAGCGCC-3′	5′-TGGATGTTCTTGAGGTGAATTCC-3′	81
Fluorescence-labeled probe (FAM)	5′-TGCCAGTGCTTGCAGACCCTGC-3′		
Fractalkine	5′-CCTGTAGCTTTGCTCATCCACTATC-3′	5′-TCCAAGATGATTGCGCGTT-3′	68
Fluorescence-labeled probe (FAM)	5′-ACAGAACCAGGCATCATGCGGCA-3′		

Primers were designed using the HUSAR program. Each sequence was validated by comparing it against the NCBI database.

tions (1:10, 1:100, 1:1000) were prepared from each cDNA sample and *GAPDH* was amplified. Expression levels of all other genes are given relative to the expression levels of *GAPDH* by evaluation of their crossing-over points of product accumulation curves relative to the standard curve of *GAPDH.* All PCR products were checked by melting point analysis and by gel electrophoresis to verify that products were of the correct lengths. Several of the PCR products were cycle-sequenced to confirm their identity.

Laser-Assisted Microdissection for Oligonucleotide Array

Microdissection was performed using P.A.L.M. microlaser technology (P.A.L.M. GmbH, Bernried, Germany) on frozen sections stained by hematoxylin to obtain ~20,000 pooled epithelial and stromal cells each from five histological normal (N2, N4, N5, N6, N7) and five cancerous samples (T8, T9, T10, T14, T17). For the normal epithelium the procedure was performed twice to hybridize the resulting labeled cRNA to two different chips and determine reproducibility. Total RNA was extracted by the Chomczynski and Sacchi[13] method as above; however, in view of the small amounts of total RNA expected, 2 μl of Pellet Paint (Novagen, Darmstadt, Germany) were added as a co-precipitant before RNA precipitation. RNA quality was checked on RNA lab chips by the Bioanalyzer 2100 Lab-On-A-Chip system (Agilent Technologies, Palo Alto, CA) and found to be excellent, with a 2:1 ratio of 28S to 18S RNA. From peak heights obtained in a separate pilot study with 200, 100, and 20 ng of total mouse RNA, the RNA amounts were estimated at between 20 ng and 100 ng in four samples (two normal epithelium, one tumor epithelium, one tumor stroma), whereas it was ~20 ng in the normal stroma sample. These RNA amounts were amplified by the protocol of Baugh and colleagues,[14] with the following modification: a temperature of 50°C was used in the RT step (total volume 10 μl) because this resulted in a higher yield of cDNAs. This modification had been verified previously by using 8 μg (standard Affymetrix protocol) *versus* 200 or 20 ng of mouse total RNA that were amplified and hybridized to Affymetrix MG-U74A chips (M. Kenzelmann and colleagues, manuscript in preparation). The yields of biotinylated cRNAs from the microdissected samples were between 7.2 (from the lowest RNA amount) and 11.5 μg. For hybridization, 7.2 μg of each of the five samples were hybridized to Affymetrix Test3 chips, which demonstrated that the amplified cRNAs were of good quality; the samples were then hybridized to HG-U95A chips as described above.

Laser-Assisted Microdissection for Quantitative Real-Time RT-PCR

Approximately 4000 epithelial and stromal cells each were microdissected as above for RT-PCR quantification of two selected messages, the *GRO2* oncogene and fractalkine transcripts. These were quantified separately for three normal (N2, N4, N7) and three cancer samples (T8, T10, T17). The primer sequences used are given in Table 2. Reverse transcription was performed as described above; real-time quantitative RT-PCR was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) using heat-activated TaqDNA polymerase (AmpliTaq Gold, Applied Biosystems). After an initial hold of 2 minutes at 50°C and 10 minutes at 95°C the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. The cDNA content of each sample was compared with another sample following the δ-δ-Ct technique.[15,16] Similar amplification efficiencies for targets and housekeeping genes were demonstrated by analyzing serial cDNA dilutions showing an absolute value of the slope of log input cDNA amount *versus* δ-CT (=Ct > housekeeping gene − Ct target) of <0.1.

Statistics

We selected genes for clustering according to Welsh and colleagues.[11] Data from all microarrays [28 samples including 2 repetitions for testing the reproducibility of the method (N2R and T7R)] were first analyzed by Affymetrix software (Data Mining Tool) for genes with the highest standard deviation (SD) (SD > 2500). This list of genes was used in Gene Spring software (Silicon Genetics, Redwood, CA) to perform a hierarchical clustering analysis[17] without any information given on histopathology of the prostate samples. A second statistical analysis was also performed on the whole data set of the normal adjacent to cancer samples (N1 to N9) and for the 10 cancer samples assigned by clustering as most distinct from normal (T8 to T17) because both of these two groups appeared as relatively homogeneous in their gene expression patterns internally. Genes with moderate to high expression levels and with a fold change >2.5 between normal and cancer gene groups and a $P < 0.05$ by Student's *t*-test were identified. The *t*-test used the two-tailed distribution and the heteroscedastic type (two samples, unequal variance).

Results

Expression Profiles of mRNA Species from Prostate Cancer and Normal Adjacent to Cancer Samples

Tumor tissues from prostate cancer patients ($n = 17$) were compared to the histological normal tissues ($n = 9$) of prostatectomized patients. Tissues from the peripheral region were used so that comparisons between the peripheral area and central regions (where benign prostatic hyperplasia is preferentially located) would be avoided.

The reproducibility of our procedures for expression profiling on DNA microarrays was tested in two ways: first, two neighboring areas (N2 and N2R) of normal tissue from the same patient were extracted for total RNA; after conversion of both RNA samples to biotin-labeled

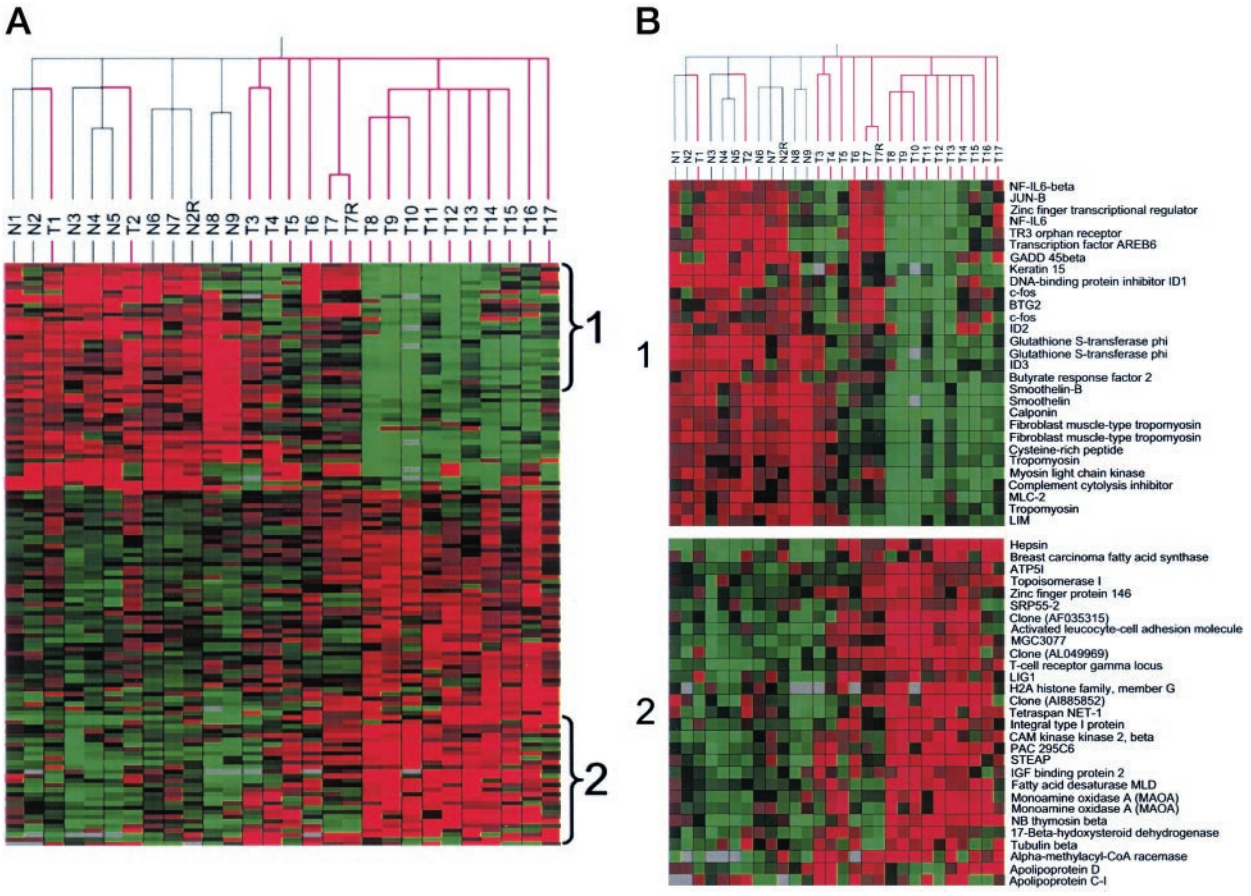

Figure 1. Hierarchical clustering analysis of the 129 genes with the highest SD. **Rows** represent individual genes; **columns** represent individual prostate samples (normal adjacent to cancer samples, N1 to N9; cancer samples, T1 to T17). Each cell in the matrix represents the expression level of a single transcript in a single sample. Genes that are up-regulated appear in red, those that are down-regulated appear in green; black, indicates approximately the same gene expression as the median for that gene across all samples. Color saturation is proportional to the magnitude of the difference from the mean. **A:** Overall view of all genes and samples after hierarchical clustering. First a list of the 129 genes with the highest SD was extracted from the whole data set (~12,600 sequences). This list was clustered by the software without any information about the nature of the samples. With the exception of two cancer samples the carcinomas (**right**) were separated from the normal group (**left**) automatically by this clustering procedure. For further analysis of the whole data set we compared the normal samples (N1 to N9) with the 10 cancer samples on the **right** in this figure (T8 to T17) because they appear relatively homogeneous in their gene expression pattern (see Tables 3 and 4). **B:** Enlarged view of areas demarcated in **A** including gene descriptions. Area 1 shows underexpressed genes, area 2 shows overexpressed genes.

cRNA according to the Affymetrix protocol and hybridization to two chips, the correlation coefficient was $r = 0.95$. Second, one-and-the-same sample from a tumor area (T7 and T7R) was also hybridized to two chips yielding a correlation coefficient of $r = 0.98$.

When the expression data from the 28 tissues (a tumor and a normal sample in duplicate, respectively) were analyzed statistically, a list of genes could be defined that showed very large standard deviations (>2500). Unsupervised clustering with this discriminatory set of sequences could distinguish between tumor and nontumor samples on the basis of gene expression patterns alone (Figure 1) with the exception of two tumors that were grouped with normal adjacent to cancer samples. In a second analysis, 10 tumor samples appearing most homogeneous in their gene expression pattern were compared to the nine normal samples; genes with a greater than 2.5-fold difference in expression were grouped; 63 genes were found with significantly increased RNA levels, and 153 genes were detected with a significant decrease in RNA levels; surprisingly the down-regulated genes were 2.4-fold more numerous than the elevated genes (Tables 3 and 4).

Tables 3 and 4 include very recently described putative marker mRNAs as well as new ones described here as significantly elevated, eg, *SGP28*, α-methylacyl-CoA racemase, *PYCR1*, and significantly down-regulated, eg, *ID1*, *ATDC*, and uteroglobin.

Confirmation of Array Data by Quantitative Real-Time RT-PCR

Eight different gene transcript species including hepsin, which has been reported previously by several investigators to be elevated in prostate cancer, were selected for confirmation of the array data by real-time RT-PCR (Figure 2). Of the eight genes chosen, five were overexpressed in prostate cancer: α-methylacyl-CoA racemase, LDL-phospholipase A2, hepsin, pyrroline 5-carboxylate reductase 1, transcriptional regulator *ERG*. The remaining three genes were underexpressed: uteroglobin (inhibitor of phospholipase), ataxia telangiectasia group D-associated protein

Table 3. Genes Overexpressed in Prostate Cancer

Accession no.*	Description	Fold change average†	Location of high gene expression‡	Normal average§	Cancer average§	*P*¶
X94323	Specific granule protein (SGP28)	21.1	Tumor epithelium	61	1277	0.0265
Z98744	H2A histone family, member D	10.4	Tumor epithelium	346	3606	0.0012
S82986	Homeo box C6	8.0	Tumor epithelium	139	1116	0.0172
AJ130733	Alpha-methylacyl-CoA racemase	6.2	Tumor epithelium	1153	7132	0.0060
U24577	LDL-phospholipase A2, group VII	6.2	Tumor epithelium	369	2281	0.0112
AB017430	Kinesin-like DNA binding protein (KNSL4)	5.6	n.s.d.	275	1549	0.0033
AD001528	Spermine synthase	5.6	n.s.d.	1364	7628	0.0087
X07732	Hepsin	5.3	Tumor epithelium	2344	12315	<0.0001
AI039144	H2A histone family, member A	5.2	Tumor epithelium	832	4308	0.0007
AF007149	Clone	5.0	Tumor epithelium	383	1924	0.0091
U80456	Transcription factor SIM2	5.0	Tumor epithelium	379	1898	0.0286
AL049977	Claudin 8	4.7	Tumor epithelium	891	4207	0.0013
M77836	Pyrroline 5-carboxylate reductase 1	4.4	Tumor epithelium	733	3244	0.0001
U83660	Multidrug resistance-associated protein (MRP4)	4.4	Tumor epithelium	270	1175	0.0456
AL049977	Claudin 8	4.3	Tumor epithelium	390	1665	0.0078
AB002387	Myosin VI	4.2	Tumor epithelium	351	1482	0.0024
AF071202	ABC transporter MOAT-B	4.1	Tumor epithelium	470	1922	0.0237
X92689	GalNac-T3	3.9	Tumor epithelium	295	1155	0.0362
AF052107	Clone	3.9	n.s.d.	1158	4526	0.0001
M99487	Prostate-specific membrane antigen 1 (PSMA)	3.9	Tumor epithelium	2867	11194	0.0219
AI936759	Clathrin coat associated protein AP19	3.8	Tumor epithelium	330	1255	0.0039
X00088	H2B histone family, member R	3.8	Tumor epithelium	307	1164	0.0224
AI189287	H1 histone family, member 2	3.8	Tumor epithelium	599	2247	0.0024
AF007216	Sodium bicarbonate cotransporter (HNBC1)	3.7	Tumor epithelium	1802	6656	0.0018
AA290994	Clone	3.7	n.s.d.	446	1649	0.0383
D82345	NB thymosin beta	3.6	Tumor epithelium	2317	8232	<0.0001
AL080199	Clone	3.4	Tumor epithelium	385	1327	0.0028
M95610	Alpha 2 type IX collagen (COL9A2)	3.3	Tumor epithelium	459	1495	0.0113
L08044	Human intestinal trefoil factor 3	3.2	Tumor epithelium	3807	12338	0.0469
AL049764	Peroxisomal membrane protein (PMP34)	3.2	n.s.d.	337	1092	0.0001
AL079298	Methylcrotonoyl-CoA carboxylase 2	3.2	Tumor epithelium	545	1733	0.0243
AI620381	MGC3077	3.1	n.s.d.	3711	11604	0.0004
AL049969	Clone	3.1	n.s.d.	5865	18178	0.0008
M30894	T-cell receptor gamma locus	3.1	Tumor epithelium	6539	19975	<0.0001
AI935146	GalNac-T3	3.0	Tumor epithelium	592	1775	0.0015
Y10183	Activated leukocyte-cell adhesion molecule	3.0	Tumor epithelium	2182	6494	0.0026
M99487	Prostate-specific membrane antigen 1 (PSMA)	2.9	Tumor epithelium	2504	7307	0.0230
X87176	17-Beta-hydroxysteroid dehydrogenase 4	2.9	n.s.d.	1908	5557	0.0083
AF045229	Regulator of G protein signaling 10 (RGS10)	2.9	n.s.d.	405	1165	0.0001
AF035315	Clone	2.9	Tumor epithelium	2523	7194	0.0002
AB011004	UDP-N-acetylglucosamine pyrophosphorylase	2.8	n.s.d.	1058	2984	<0.0001
D87682	KIAA0241	2.8	n.s.d.	395	1108	0.0001
U51903	RasGAP-related protein (IQGAP2)	2.8	Tumor epithelium	965	2699	0.0004
AC005053	STEAP	2.8	Tumor epithelium	5785	16147	0.0001
AI885852	H2A histone family, member O	2.8	Tumor epithelium	8332	23201	0.0006
AI570572	Ras-related C3 botulinum toxin substrate 3	2.8	n.s.d.	590	1636	0.0050
AB017563	Immunoglobulin superfamily, member 4	2.7	Tumor epithelium	1249	3417	0.0499
Z80780	H2B histone family, member H	2.7	Tumor epithelium	893	2429	0.0096
M68840	Monoamine oxidase A (MAO A)	2.7	n.s.d.	2421	6555	0.0065
AL109672	Integral type I protein	2.7	Tumor epithelium	3681	9922	0.0019
AL049933	G protein, alpha-1 subunit	2.7	n.s.d.	564	1496	<0.0001
X73424	Propionyl-CoA carboxylase a subunit	2.6	n.s.d.	822	2170	0.0005
AC002073	LIM domain kinase 2	2.6	Tumor epithelium	826	2173	<0.0001
Z80776	H2A histone family, member G	2.6	Tumor epithelium	2525	6627	0.0015
L41816	Cam kinase I	2.6	n.s.d.	633	1650	0.0243
AI200373	H2A histone family, member I	2.6	Tumor epithelium	938	2439	0.0032
M93036	Carcinoma-associated antigen GA733-2	2.6	Tumor epithelium	2741	7010	<0.0001
AL080181	Immunoglobulin superfamily, member 4	2.6	n.s.d.	2053	5236	0.0087
AB018330	CAM kinase kinase 2, beta	2.5	Tumor epithelium	2710	6900	0.0023
L10333	Reticulon 1	2.5	Tumor epithelium	412	1040	0.0065
AF002668	Fatty acid desaturase MLD	2.5	n.s.d.	4574	11501	0.0018
AL009179	H2B histone family, member C	2.5	Tumor epithelium	1004	2522	0.0086
AC004381	Clone	2.5	Tumor epithelium	681	1710	0.0468

*GenBank accession number.

†Fold change represents mRNAs differentially up-regulated in carcinoma relative to adjacent normal tissue.

‡Putative cell-type location of high gene expression as suggested of selected microdissected material. Only fold differences >4 between gene expression in tumor epithelium and tumor stroma were taken into consideration.

§Average, average difference = expression intensity calculated by Affymetrix software.

¶*P* value was calculated by the Student's *t*-test.

n.s.d., no significant difference, i.e. fold difference between gene expression in tumor epithelium and tumor stroma <4.

Some genes are represented by several sequences on the microarray, e.g. Claudin 8, GalNac-T3, PSMA.

Table 4. Genes Underexpressed in Prostate Cancer (50 of 153 Found Genes Are Shown)

Accession no.*	Description	Fold change average†	Location of high gene expression‡	Normal average§	Cancer average§	*P*¶
L15702	Complement factor B	−11.9	n.s.d.	3350	282	0.0284
AI762213	Lipocalin 2	−9.7	Normal epithelium	3614	372	0.0309
AF022991	Period circadian protein 1 (RIGUI)	−9.6	n.s.d.	2252	234	0.0011
M36820	GRO-2 oncogene	−9.3	n.s.d.	2389	258	0.0106
X63187	WAP four-disulfide core domain 2 (HE4)	−7.9	n.s.d.	3358	423	0.0006
Z71929	Fibroblast growth factor receptor 2	−7.9	n.s.d.	1083	137	0.0011
D87463	KIAA0273	−7.6	n.s.d.	1188	156	<0.0001
D10667	Smooth muscle myosin heavy chain MYH11	−7.4	Normal STROMA	10632	1442	0.0003
M60278	Heparin-binding EGF-like growth factor	−7.3	n.s.d.	1831	252	0.0452
AL050138	Elastin microfibril interface located protein	−7.1	Normal STROMA	1264	178	<0.0001
AW003733	Rho-related protein HP1	−6.5	Normal STROMA	1157	179	0.0116
S78825	DNA-binding protein inhibitor ID1	−6.3	Normal epithelium	2026	324	0.0212
M21389	Keratin 5	−6.0	Normal epithelium	6708	1123	0.0024
AI887421	RAR-responsive protein TIG1	−5.4	n.s.d.	1330	245	0.0165
L24203	ATDC	−5.4	Normal epithelium	3108	575	0.0134
U84487	Fractalkine	−5.2	n.s.d.	2584	494	0.0029
W28589	Heat shock protein HSP60	−5.0	Normal STROMA	1991	397	<0.0001
J04102	ETS-2	−5.0	n.s.d.	2564	517	0.0067
U95626	Chemokine receptor 2	−5.0	Normal epithelium	10851	2189	0.0036
AF023614	Transmembrane activator and CAML interactor	−4.9	n.s.d.	2210	448	<0.0001
D15050	Transcription factor AREB6	−4.7	n.s.d.	6626	1405	0.0044
X08020	Glutathione *S*-transferase subunit 4	−4.6	n.s.d.	2670	579	0.0103
L13698	Growth arrest-specific 1 (GAS1)	−4.5	n.s.d.	2360	524	0.0006
S77154	NR4A2	−4.5	Normal STROMA	1429	317	0.0113
M12174	RhoB	−4.4	n.s.d.	2007	452	0.0380
U27185	RAR-responsive protein TIG1	−4.4	n.s.d.	1788	406	0.0180
AI888563	Smoothelin	−4.3	n.s.d.	6850	1602	0.0001
X07696	Keratin 15	−4.2	Normal epithelium	6401	1508	0.0017
L49169	FosB	−4.2	n.s.d.	9912	2336	0.0020
D10667	Smooth muscle myosin heavy chain	−4.2	n.s.d.	12532	2956	<0.0001
L19871	Activating transcription factor 3 (ATF3)	−4.2	n.s.d.	5372	1270	0.0119
J00073	Alpha-cardiac actin gene	−4.2	Normal STROMA	2197	525	0.0015
AJ012737	Filamin C, gamma	−4.1	Normal STROMA	3878	956	<0.0001
M69225	Bullous pemphigoid antigen 1 (BPAG1)	−4.0	Normal epithelium	2327	575	0.0053
AF017257	ETS-2	−4.0	n.s.d.	1394	346	0.0054
Y16961	Tumor protein p63	−4.0	Normal epithelium	1982	494	0.0006
AA149644	Clone	−4.0	Normal STROMA	1315	329	0.0032
D84110	RBP-MS/type4	−4.0	n.s.d.	2189	548	<0.0001
L13698	Growth arrest-specific 1 (GAS1)	−4.0	n.s.d.	2691	674	0.0009
U25138	MaxiK potassium channel beta subunit	−4.0	Normal STROMA	4719	1186	0.0001
AJ238246	Keratin 7 (sarcolectin)	−3.9	Normal epithelium	1264	321	0.0482
X54162	Leiomodin 1 (smooth muscle)	−3.9	Normal STROMA	6099	1558	0.0006
M24736	Selectin E	−3.9	Normal STROMA	1727	445	0.0115
AB007836	Hic-5	−3.9	Normal STROMA	3255	840	<0.0001
Y13492	Smoothelin-B	−3.8	Normal STROMA	13313	3469	<0.0001
T92248	Uteroglobin	−3.8	Normal epithelium	2129	562	0.0342
X93498	21-Glutamic acid-rich protein	−3.8	Normal STROMA	1181	312	0.0388
X53416	Filamin A, alpha	−3.8	n.s.d.	3064	814	0.0019
AB002351	KIAA0353 gene	−3.7	Normal STROMA	10126	2720	0.0002
U15932	Dual-specificity protein phosphatase	−3.7	n.s.d.	2142	580	0.0363

*GenBank accession number.
†Fold change represents mRNAs differentially down-regulated in carcinoma relative to adjacent normal tissue.
‡Putative cell-type location of high gene expression as suggested of selected microdissected material. Only fold differences >4 between gene expression in normal epithelium and normal stroma were taken into consideration.
§Average, average difference = expression intensity calculated by Affymetrix software.
¶*P* value was calculated by the Student's *t*-test.
n.s.d., no significant difference, i.e. fold difference between gene expression in normal epithelium and normal stroma <4.
Some genes are represented by several sequences on the microarray, e.g. TIG1, ETS-2, GAS1.

(*ATDC*), and DNA-binding protein inhibitor *ID1.* For all increased RNAs, corroboration by real-time PCR was obtained (correlation coefficients of $r = 0.72$ to 0.96) indicating quantitative agreement between oligonucleotide array data and RT-PCR data. Decreased RNA results also were confirmed for uteroglobin and *ATDC*, whereas *ID1* was decreased in three of five cancer samples with an overall correlation coefficient of $r = 0.52$.

Expression Profiles from Microdissected Samples

To achieve an analysis of up- or down-regulated genes not only in bulk tissue but also in epithelial or stromal cells of tumors and of normal tissue adjacent to cancer, laser-assisted microdissection was performed on cancerous

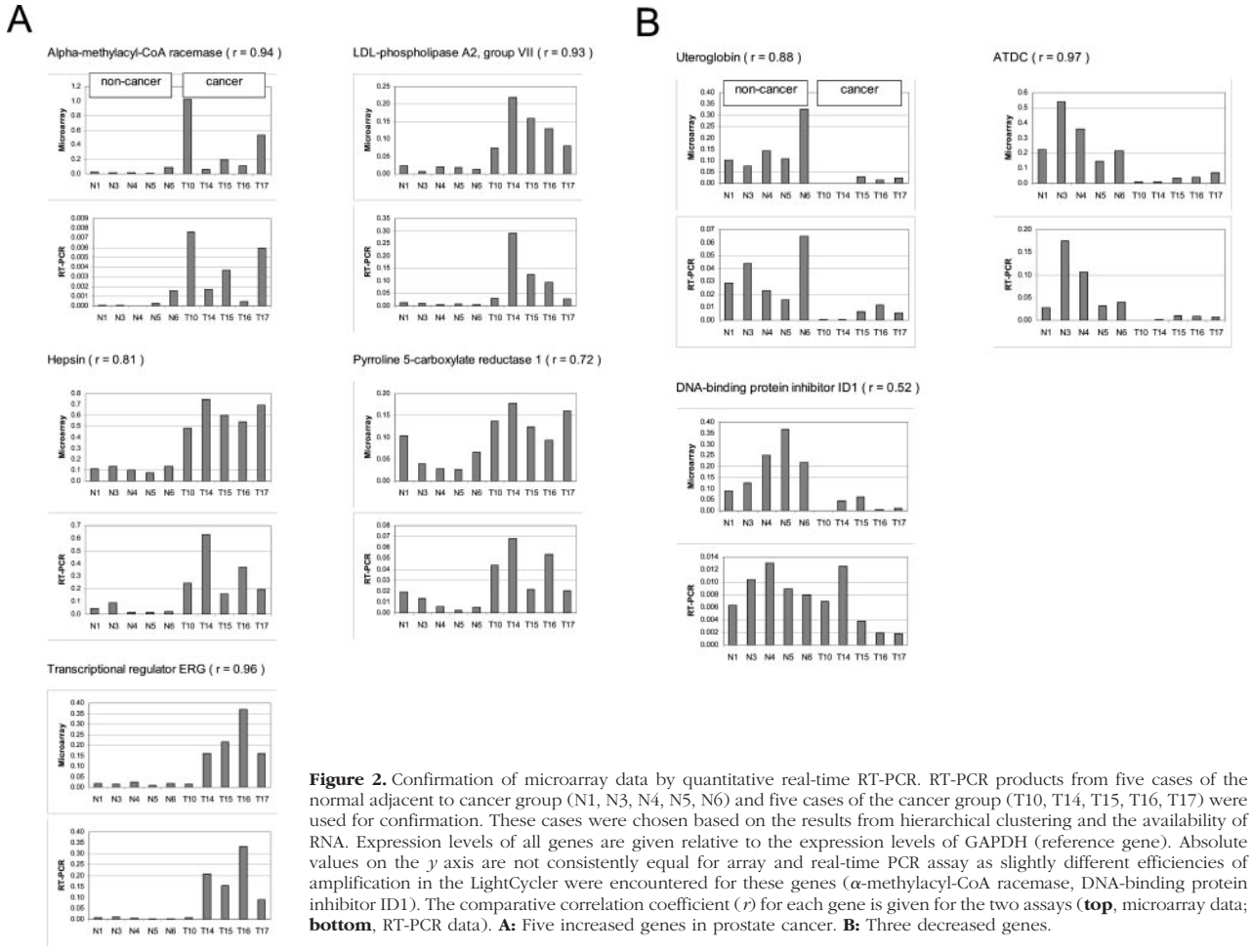

Figure 2. Confirmation of microarray data by quantitative real-time RT-PCR. RT-PCR products from five cases of the normal adjacent to cancer group (N1, N3, N4, N5, N6) and five cases of the cancer group (T10, T14, T15, T16, T17) were used for confirmation. These cases were chosen based on the results from hierarchical clustering and the availability of RNA. Expression levels of all genes are given relative to the expression levels of GAPDH (reference gene). Absolute values on the *y* axis are not consistently equal for array and real-time PCR assay as slightly different efficiencies of amplification in the LightCycler were encountered for these genes (α-methylacyl-CoA racemase, DNA-binding protein inhibitor ID1). The comparative correlation coefficient (*r*) for each gene is given for the two assays (**top**, microarray data; **bottom**, RT-PCR data). **A:** Five increased genes in prostate cancer. **B:** Three decreased genes.

glands and stromal areas, respectively, from five advanced tumors (T8, T9, T10, T14, T17) and glands and stroma from five normal adjacent to cancer tissues (N2, N4, N5, N6, N7). The excised areas were pooled and extracted for RNA as described before; RNAs were tested for their quality on RNA electrophoresis chips and showed no evidence of RNA degradation. Between 20 and 100 ng of RNA were amplified by the protocol of Baugh and colleagues[14] with minor modifications. When the two pools of normal epithelia (NE1, NE2) were compared on oligonucleotide microarrays the correlation coefficient was $r = 0.95$ indicating excellent reproducibility of the method. Evaluation of the three pools of normal [two normal epithelial cells (NE1, NE2), one normal stroma] and separately of the two pools of tumor tissue [tumor epithelial cells (TE) and tumor stromal cells] indicated that many of the genes were differentially expressed (by at least fourfold) in epithelial *versus* stromal cells (see Tables 3 and 4). A comparison of the tumor epithelium microarray (TE) with the two normal epithelium microarrays (NE1, NE2) is given in Table 5 for genes with a greater than 15-fold difference in gene expression.

To corroborate the microarray data from microdissected epithelial and stromal cells real-time PCR of cDNA products from epithelial and stromal cells from three tumor (T8, T10, T17) and three normal samples (N2, N4, N7) was performed for the messages of two chemokines, *GRO2* and fractalkine. In addition to their chemotactic properties on mononuclear cells, *GRO2* and fractalkine are thought to possess mitogenic/oncogenic (*GRO2*) and anti-apoptotic activities (fractalkine).[18,19] *GRO2* and fractalkine mRNAs were down-regulated in tumor epithelium and surrounding stroma (Figure 3).

Cytogenetic Position of Overexpressed or Underexpressed Genes

Several genes over- or underexpressed in prostate cancer were found to be in close genomic proximity. Four exemplary bands are shown for chromosomes 6, 7, and 21 (Table 6).

Discussion

The gene expression analysis on 17 prostate carcinomas revealed several genes that showed increases in expression of up to 21-fold (ie, specific granule protein *SGP28*) and decreases of up to 12-fold (ie, complement factor B) in comparison to adjacent nontumorous tissue (Tables 3 and 4). These genes, some of which have already been

Table 5. Comparison of Microdissected Tumor Epithelial Areas with Microdissected Normal Epithelial Areas

Accession no.*	Description	Fold change† TE/NE1	Fold change‡ TE/NE2
Overexpressed genes			
X96584	NOV (nephroblastoma overexpressed gene)	63.4	16.6
M94856	Fatty acid binding protein 5	42.1	28.6
M21535	Transcriptional regulator ERG	35.5	35.5
U21128	Lumican	34.7	18.1
J03870	Cystatin SN	26.2	25.6
AL049977	**Claudin 8**	24.7	24.6
X94323	**Specific granule protein (SGP28)**	20.0	19.1
X07820	Matrix metalloproteinase 10 (stromelysin 2)	19.6	18.5
AJ006835	RNU17D	17.8	18.5
AF053356	Clone	17.3	16.7
Underexpressed genes			
U95626	**Chemokine receptor 2**	−128.1	−42.8
AF022991	**Periodic circadian protein 1 (RIGUI)**	−54.0	−70.6
AB006532	RecQ protein-like 4	−42.2	−18.2
Z49878	Guanidinoacetate N-methyltransferase	−30.9	−19.2
AB018278	Synaptic vesicle protein 2B homolog	−20.9	−15.5
M21389	**Keratin 5**	−19.3	−17.4
U17760	Laminin, beta 3	−18.6	−19.7
AJ238246	**Keratin 7 (sarcolectin)**	−15.2	−18.9
AF085807	Uroplakin IA	−15.0	−15.3

*GenBank accession number.
†Fold change represents mRNAs differentially over- or underexpressed in microdissected tumor epithelium (TE) relative to microdissected normal epithelium (NE1, NE2). Only fold differences >15 were taken into consideration.
‡Fold change was calculated by Affymetrix software.
Transcripts in bold letters were also found as significantly over- or underexpressed in bulk prostate tissue (see Tables 3 and 4).

described, may serve as markers of prostate carcinoma. Our data on total prostate tissue confirm and extend the findings of recently published microarray studies using oligonucleotide array technology.[8–11,20] Our study confirms a statistically significant overexpression of the gene for hepsin (*HPN*, X07732, a transmembrane serine protease) in the majority of tumors (Table 3). Overexpression of the message for pyrroline-5-carboxylate reductase 1 (*PYCR1*, M77836) was reported before as a p53-dependent protein in a DNA microarray study on cultured tumor cells,[21] and we now have found this message overexpressed in prostate cancer. It is of note that the compound pyrroline-5-carboxylate 1 induced apoptosis in cultured cells overexpressing *PYCR1.*[21] Prostate-specific membrane antigen, a novel folate hydrolase associated with prostatic carcinogenesis and metastasis,[22] also was up-regulated in tumor epithelium in our study. Of particular note in the present study is that down-regulated genes may be at least as informative as up-regulated genes in characterizing prostate cancer. Loss or a decrease of gene expression may be relevant in early prostate cancers, that were studied. This corresponds to results in cytogenetic studies that have described preferentially losses of genes in early stages of prostate cancer.[23,24] Down-regulation of certain genes may prove to be directly involved in fostering tumor development or metastasis. Bullous pemphigoid antigen 1 is a component of hemidesmosome plaque 1; its reduction may loosen epithelial adherence. The mRNAs of cytoskeletal proteins such as keratins 5, 15, and 7 as components of basal cells, which are not part of atypical prostatic epithelium, were significantly down-regulated.

In addition, a novel aspect of this report is the microarray analysis on microdissected tissues and the usefulness of this approach in revealing compartment-specific expression profiles. When the new protocol for amplification of ng amounts of total RNA was tested for consistency using 10 μg *versus* 20 ng of mouse thymus RNA, only 230 of 12,600 (~2%) of the sequences behaved as true outliers (at least fourfold change). Several of the overrepresented sequences were short and contained repetitions of A-rich segments (M. Kenzelmann and colleagues, in preparation). Microdissection gene expression analysis can potentially be done on prostate biopsies as the described RNA amplification procedure can be performed on a few hundred cells. This approach would divulge the gene expression pattern of tumor cells without fibromuscular tissue and without inflammatory mononuclear cells. It thus might be feasible to define differences in marker gene expression also between prostate intraepithelial neoplasia and prostate carcinoma; this may provide supplementary information on the pathogenesis of precancerous prostate lesions.

Gene expression analysis alone cannot provide an overall integrative molecular understanding of the genesis and growth of prostate carcinoma because chromosomal aberrations, translational control of messages, and posttranslational modifications, to name just a few molecular events, certainly play a major role in the biological behavior of prostate carcinoma. Table 6 shows chromosomal locations of some genes that were found to be significantly up- or down-regulated in our study. At four chromosomal locations 6p21, 7p14, 7q21, and 21q22, aberrantly regulated genes were densely concentrated. The dense location of genes with altered expression in prostate cancer at one site of chromosome 21 is interesting because this chromosome has the lowest gene density of all chromosomes. These gene expression hot

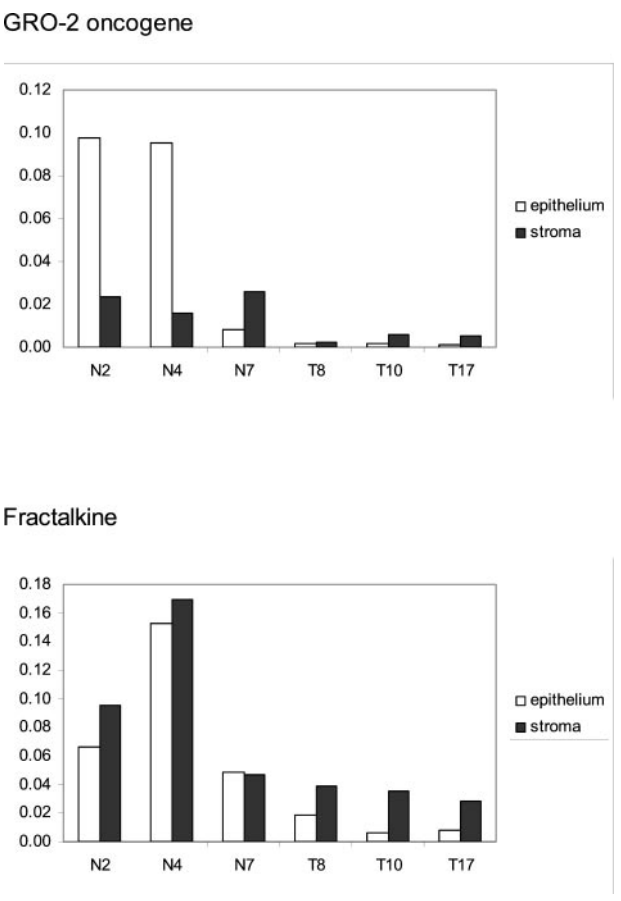

Figure 3. Quantitative real-time RT-PCR of microdissected samples for two down-regulated genes. Approximately 4,000 epithelial and stromal cells each were microdissected and RT-PCR products were quantified separately for three normal adjacent to cancer (N2, N4, N7) and three cancer samples (T8, T10, T17). Expression levels are given relative to the expression levels of GAPDH (reference gene). The Gro-2 and fractalkine genes were found significantly decreased in the microarray assay (see Table 4). Here we investigated if this down-regulation mainly occurs in epithelial or stromal cells. Gro-2 and fractalkine mRNA seem to be down-regulated in both tumor epithelium and surrounding stroma.

spots seem to overlap for chromosome 7 with the chromosomal positions found to be amplified or deleted by comparative genomic hybridization; there are no similarities in location with comparative genomic hybridization for the other two chromosomes 6 and 21. Molecular cytogenetic analysis has identified common sites of chromosomal alterations in prostate cancer: gains at 7p, 7q, 8q, Xq, and losses at 5q, 6q, 8p, 13q, 16q, and 18q.[23–26]

New hypotheses on prostatic carcinogenesis may be entertained by inspection of the gene expression changes identified in the current study, in particular by the array data from microdissected epithelial and stromal cells. Apart from messages for putative new markers mentioned before, several themes emerged from the compilation of up- and down-regulated genes: first, the up-regulated expression of several members of the histone family (Tables 3 and 6) we observed may indicate a dysregulation of chromatin structure.[27] Second, transcription factors and growth factors were not uniformly up-regulated. Several transcription factors/activators and growth factors actually were clearly down-regulated (Table 4). Also the down-regulation of suppressor and growth arrest genes can be seen as particularly important in the pathogenesis of early prostate cancers that were studied. As varying numbers of mononuclear infiltrates can be found in prostate carcinoma, it was expected that stromal and/or epithelial cells might express chemokines. For example, surprisingly the mRNA of chemokine *GRO2* (growth-related oncogene) and of the cell-bound CX_3C chemokine fractalkine were found decreased as well as the messages for chemokine receptor 2, receptor for the β-chemokine MCP-1. Besides their role in the induction and propagation of inflammatory reactions the two tested chemokines can exhibit growth factor-like (*GRO2*) and anti-apoptotic (fractalkine) activities.[18,19] The decrease of *GRO2* and fractalkine was observed in tumor epithelium and tumor stroma, as revealed by microdissection and real-time PCR. This also shows that the observed down-regulation of genes in bulk tumor tissue cannot simply be explained by the nonpresentation of stromal genes in tumors that have a relative low stroma content. Third, several mRNAs coding for proteins in fat and steroid metabolism were dysregulated in prostatic carcinoma in this study, including up-regulation of α-methylacyl-CoA racemase[10] that catalyzes the degradation of branched fatty acids and C27 steroids[28] and of LDL phospholipase A2 that generates short fatty acids (up-regulated sixfold). Fatty acid metabolism and steroids have been implicated in prostate carcinogenesis. Enzymes in fatty acid synthesis have been proposed as targets for anti-neoplastic therapy.[29,30] Uteroglobin (a polychlorinated biphenyl-binding protein) was found down-regulated in the prostate carcinoma samples. This protein can inhibit phospholipase A2 activity.[31] It is able to disrupt the generation of platelet-activating factor and has been reported to reduce the growth of an adenocarcinoma cell line.[32] In accordance with another report[33] the message of cyclooxygenase-2, a catalytic enzyme for the synthesis of inflammatory and carcinogenic prostaglandin derivatives, was not found up-regulated in cancerous tissue. Cyclooxygenase-2 apparently is important in the development of proliferative inflammatory atrophy.

In summary we showed here that mRNA expression analysis with microarray identified a set of genes that characterize prostate cancer and normal tissue adjacent to cancer. Down-regulated genes were found to be more numerous than up-regulated genes and are equally suited for differentiation of bulk normal from cancerous tissues. Array analysis of microdissected epithelia and stromal cells localized the majority of up-regulated genes to cancerous epithelia. These data can be used to design an array with a restricted number of cDNA or oligonucleotides for the study of larger sample sets and eventually supplementary diagnostic purposes. The current study demonstrated that even small amounts of prostate mRNA can be used for array expression profiling. Microarray studies can be performed on microdissected tissue separating tumor epithelium and stroma and may give more insight into cellular oncogenesis of the prostate.

Table 6. Cytogenetic Positions of Genes with Significantly ($P < 0.05$) Increased or Decreased Gene Expression in Prostate Cancer

Chromosome	Cytogenetic band	Accession no.	Description	Gene expression in cancer
6	6p12-p21.2	U24577	LDL-phospholipase A2, group VII	Up
	6p21	X94323	Specific granule protein (SGP28)	Up
	6p21.1-p22.2	AI039144	H2A histone family, member A	Up
	6p21.3-p22	Z98744	H2A histone family, member D	Up
	6p21.3	Z80776	H2A histone family, member G	Up
	6p22-21.3	AI200373	H2A histone family, member I	Up
	6p21.3	AI885852	H2A histone family, member O	Up
	6p22-21.3	AL009179	H2B histone family, member C	Up
	6p21.3	Z80780	H2B histone family, member H	Up
	6p21.3	X00088	H2B histone family, member R	Up
	6p21.3	AI189287	H1 histone family, member 2	Up
	6p21.3	L15702	Complement factor B	Down
7	7p14	D87682	KIAA0241	Up
	7p14-p15	AI620381	MGC3077	Up
	7p14-p15	M30894	T-cell receptor gamma locus	Up
	7q21	AI936759	Clathrin coat-associated protein AP19	Up
	7q21	AL049933	G protein, alpha-1 subunit	Up
	7q21	AC005053	STEAP	Up
21	21q22.2	J04102	ETS-2	Down
	21q22.2	U80456	Transcription factor SIM2	Up
	21q22.3	L08044	Human intestinal trefoil factor 3	Up
	21q22.11	AL049977	Claudin 8	Up

Gene locations as given in the NCBI database.
Up, gene expression significantly ($P < 0.05$) increased in prostate cancer relative to adjacent normal tissue; Down, gene expression significantly ($P < 0.05$) decreased in prostate cancer relative to adjacent normal tissue.

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