Gene Expression Profiling in Clinically Localized Prostate Cancer: A Four-Gene Expression Model Predicts Clinical Behavior

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ABSTRACT

Purpose: **New diagnostic and prognostic molecular markers are required for prostate cancer, one of the most common male malignancies in Western countries. Gene expression profiling may help to identify genes involved in prostate carcinogenesis, yield clinical biomarkers, and improve tumor classification.**

Experimental Design: **To identify fundamental differences between normal and neoplastic prostate tissue, we used real-time quantitative RT-PCR assays to quantify the mRNA expression of 291 selected genes in samples of normal prostate and of well-documented primary, clinically localized prostate tumors.**

Results: **Forty-six genes showed significantly different expression in tumors relative to normal prostate. The dysregulated genes belong notably to the extracellular membrane and extracellular membrane remodeling categories and are involved in angiogenesis. Furthermore, we obtained a four-gene (*XLKD1/LYVE1*, *CGA*, *F2R/PAR1*, and *BCL-G*) model that discriminated between the seven patients with and the seven patients without relapse, independently of stage and grade.**

Conclusions: **Some dysregulated genes are good candidates for use as molecular markers and/or therapeutic targets. Furthermore, differential gene expression profiling of clinically localized prostate tumors from relapsing and nonrelapsing patients identified a set of four genes with a pattern of expression that defines a molecular signature that could predict the clinical behavior of this disease.**

INTRODUCTION

CaP[7] is one of the most common malignancies and the second cause of cancer death among Western men (1). Its morbidity and mortality in the growing elderly population makes CaP a major public health problem. Treatment is ineffective in advanced stages, and the disease must, therefore, be diagnosed early, when the tumor is still confined to the prostate and can be eradicated by radical prostatectomy. Early diagnosis of CaP is currently based on a combination of digital rectal examination and PSA assay and is confirmed by biopsy. However, these tests lack sensitivity, and radical prostatectomy carries a substantial risk of incontinence and impotence. Moreover, about one-third of men who undergo radical prostatectomy for localized tumors relapse, and this risk cannot be accurately predicted by using available markers (clinical stage, serum PSA level, Gleason score, pathological stage, and the number of positive biopsies). New diagnostic and prognostic markers and new therapeutic targets are urgently needed.

The initiation and progression of CaP involves multiple changes in gene expression. cDNA microarray technology offers the possibility of comparing the expression of large numbers of genes between normal and malignant tissues and was recently used to identify disease-related gene expression patterns in prostate samples (2–4). Discrepancies among reported CaP gene expression signatures could be attributable to the fact that prostate tumors contain many cell types, in addition to carcinoma cells, such as epithelial cells, stroma cells, endothelial cells, adipose cells, and infiltrating lymphocytes. cDNA microarray studies require abundant starting material, and neoplastic epithelial cells cannot be precisely microdissected from their normal counterparts. In addition, although cDNA microarrays can be used to compare the mRNA expression of thousands of genes analyzed in parallel, they cannot discriminate between gene clusters with a high degree of homology (*e.g.*, p14, p15, and p16 on chromosome arm 9p). Finally, cDNA microarrays cannot reveal small variations in gene expression or changes in important but weakly expressed genes (*e.g.*, *CYP19* and *hTERT* in prostate tumors).

To circumvent the technical problems inherent in the cDNA microarray technique, we used a quantitative real-time RT-PCR assay that is a reference in terms of its performance, accuracy, sensitivity, wide dynamic range, and high throughput capacity for nucleic acid quantification.

In this study, we applied RT-PCR to 291 selected genes involved in pathways (*e.g.*, cell cycling, signal transduction, apoptosis, angiogenesis, and metastasis) that are known to be

Received 2/24/03; revised 7/22/03; accepted 7/22/03.
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[7] The abbreviations used are: CaP, prostate cancer; Ct, threshold cycle; PSA, prostate-specific antigen; ROC, receiver operating characteristic; AUC, area(s) under the curve; ECM, extracellular membrane; MMP, matrix metalloproteinase;

Extracellular matrix	
CLDN4	Claudin 4
COL1A1	Collagen, type I, alpha 1
COL1A2	Collagen, type I, alpha 2
FN1	Fibronectin 1
HAS2	Hyaluronan synthase 2
HPSE	Heparanase
KAI1	kangai 1
PLG	Plasminogen
SDC1	Syndecan 1
SPARC	Secreted prot. acidic cyst-rich
SPARCL1	SPARC-like 1 (Hevin)
THBS1	Thrombospondin-1
VTN	Vitronectin
Matrix proteases	
MMP2	Matrix metalloproteinase 2
MMP7	Matrix metalloproteinase 7
MMP9	Matrix metalloproteinase 9
MMP14	MT1-MMP
PLAT	Plasminogen activator, tissue
PLAU	Plasminogen activator, urokinase
Inhibitors of matrix proteases	
SERPINB1	Serine proteinase inhibitor (PAI1)
SERPINB2	Serine proteinase inhibitor (PAI2)
TIMP1	Tissue inhibitor 1 of MMP
TIMP2	Tissue inhibitor 2 of MMP
TIMP3	Tissue inhibitor 3 of MMP
TIMP4	Tissue inhibitor 4 of MMP
Cell adhesion and cell junction	
ANXA6	Annexin A6
GJA1	Connexin 43
GJA5	Connexin 40
GJB1	Connexin 32
GJB2	Connexin 26
DSG2	Desmoglein 2
CDH1	E-Cadherin
CD44	Hyaluronan receptor (CD44 Ag)
HMMR	Hyaluronan receptor (RHAMM)
ITGA2	Integrin a1
ITGA1	Integrin a2
ITGA5	Integrin a5
ITGA6	Integrin a6
ITGAV	Integrin aV
ITGB1	Integrin b1
ITGB3	Integrin b3
ITGB4	Integrin b4
Cytoskeletal	
ACTN4	Actinin, alpha 4
CALD1	Caldesmon 1
KRT19	Keratine 19
MAPT	Microtubule-associated protein tau
TIAM1	T-cell lyphoma invasion metastasis
TMSB4X	Thymosin beta 4
TPM1	Tropomyosin 1
TUBB	Tubulin, beta polypeptide
VASP	Vasodilatator stimulated P protein
Nuclear receptors	
AR	Androgen receptor
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
PPARG	Peroxisome prolif activ Receptor g
PGR	Progesterone receptor
RARA	Retinoic acid receptor alpha
RXRA	Retinoic X receptor alpha
THRA	Thyroid hormone receptor alpha
THRB	Thyroid hormone receptor beta

Signal transduction	
APC	Adenomatosis polyposis coli
ARHA	Rho A
ARHC	Rho C
AXIN1	Axin
BCAR1	Cas
CAV1	Caveolin 1
CAV2	Caveolin 2
CD9	CD9 antigen (p24)
CDC42	Cell division cycle 42
CHC1L	Chromosome condensation 1-like
CTNNA1	Catenin alpha 1
CTNNB1	Catenin beta 1
FYN	FYN oncogene
GNB2L1	Guanine nucleotide binding protein
GRB2	Growth factor bound protein
HMG1	High mobility group 1
HRAS	H-Ras oncogene
ILK	Integrin-like kinase
IQGAP1	IQ GTPase activator 1
IQGAP2	IQ GTPase activator 2
KIS	Kinase interacting gene
MADH4	Mothers against decapentaplegic homolog 4
MAPK4	Mitogen-activated protein kinase 4
MUC1	Mucin 1
NF1	Neurofibromin
NF2	Neurofibromin 2 Merlin
NRAS	N-Ras oncogene
PAK1	p21/CDC42/RAC1 activated kinase1
PIK3CA	PI3K catalytic p110 alpha
PIK3R1	PI3K p85 alpha subunit
PIM1	Pim-1 oncogene
PLA2G1B	Phospholipase A2, group IB
PRKCA	Protein kinase C alpha
PRNP	Prion protein 2
PTEN	Phosphatase and tensin homolog
PTK2	Focal adhesion kinase (FAK)
RAB13	RAB13, member RAS oncogene family
RAC1	Rac 1
RAF1	Raf oncogene
RAI3	Retinoic acid induced 3
RASA1	GTPase activating protein 1
ROCK1	Rho-associated protein K 1
SHC1	Shc transforming protein
SOS1	Sos protein
SRC	SRC oncogene
SSI-1	Suppressor cytokine sig.1 (SOCS1)
SSPN	K-Ras oncogene
STMN1	Stathmin 1 / Oncoprotein 18
STMN2	Stathmin-like 2
STMN3	Stathmin-like 3
VIL2	Villin 2 (Ezrin/EMR)
Cell cycle	
ARF	p19/AKT protein
ATM	Ataxia telangiectasia mutated
CCND1	Cyclin D1
CCNE1	Cyclin E1
CDKN1B	CDK inhibitor 1B, p27 protein
CDKN2A	CDK inhibitor, p16 protein
CDKN2B	CDK inhibitor, p15 protein
GADD45A	GADD45 alpha
MAD2L1	Mitotic arrest deficient like 1
MDM2	Mdm2 protein
MKI67	Proliferation-related Ki-67 antigen
TOP2A	Topoisomerase II alpha
RB1	Retinoblastoma
SFN	Stratifin

Transcription factors	
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
CEBPA	CCAAT/enhancer binding prot.a
E2F1	E2F transcription factor 1
JUN	jun proto-oncogene
JUNB	jun B proto-oncogene
UND	jun D proto-oncogene
MYC	c-Myc oncogene
NKX3A	NK3 transcription factor homolog A
SNAI1	Snail homolog 1
SNAI2	Snail homolog 2 (SLUG)
TCF1	Hepatic nuclear factor 1 (HNF1)
TCF4	Transcription factor 4
Growth factors	
AREG	Amphiregulin
AS3	androgen-induced prostate associated protein
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
BMP6	Bone morphogenetic protein 6
BMP7	Bone morphogenetic protein 7
CGA	Glycoprot. hormone alpha polypeptide
CTGF	Connective tissue growth factor
EGF	Epidermal growth factor
EREG	Epiregulin
FGF2	Fibroblast growth factor 2
FGF7	Fibroblast growth factor 7
FST	Follistatin
GRO1	Gro1 oncogene
GRP	Gastrin-releasing peptide
HGF	Hepatocyte growth factor
IFNG	Interferon, gamma
IGF2	Insulin-like growth factor 2
IGFBP2	IGF binding protein 2
IL1A	Interleukin 1, alpha
IL6	Interleukin 6
IL10	Interleukin 10
INSL6	Insulin-like 6
KISS1	Kiss-1 metastasis-suppressor
KITLG	Stem cell factor
NRG1	Neuregulin 1
NRG2	Neuregulin 2
NRG3	Neuregulin 3
NTN1	Netrin 1
PDGFA	PDGF alpha chain
PDGFB	PDGF beta chain (Sis)
RLN1	Relaxin 1
SDF1	CXC chemokine ligand 12 (CXCL12)
SLIT1	Slit homolog 1
SLIT2	Slit homolog 2
SLIT3	Slit homolog 3
TGFA	Transforming growth factor, alpha
TGFB1	Transforming growth factor beta 1
TGFB2	Transforming growth factor beta 2
TNF	Tumor necrosis factor

Fig. 1 List of the selected genes.

dysregulated in solid tumors, including CaP (Fig. 1). We studied 14 clinically localized prostate tumors from well-documented sources and 7 normal prostate tissues. Our aim was to identify new candidate diagnostic and prognostic markers and therapeutic targets.

MATERIALS AND METHODS

Patients and Samples

Primary prostate tumor samples were obtained from patients undergoing prostate surgery at St. Louis Hospital (Paris), La Cavale Blanche Hospital (Brest), and Nancy University Hospital, France.

Clinically localized tumors were removed by radical prostatectomy. The surgical specimens were first sliced thickly, and samples were then cut from suspect areas.

Tissue Sample Selection

Suspect areas were stained with H&E for histopathological examination in the surgery suite, and a thick shave of an adjacent section was immediately frozen at −80°C for RNA

Growth factor receptors	
CXCR4	Chemokine (C-X-C) receptor 4
DCC	Deleted in colorectal carcinoma
EDNRB	Endothelin receptor type B
EGFR	EGF receptor (ERBB1)
ERBB2	c-erbb-2
ERBB3	c-erbb-3
ERBB4	c-erbb-4
FGFR1	Fibroblast growth factor receptor 1
FGFR2	Fibroblast growth factor receptor 2
FSHR	Follicle stimulating hormone receptor
GPR54	Gprot-coupled receptor 54 (KISSR)
GRPR	Gastrin-releasing peptide receptor
HTR2B	Serotonin 5 hydroxy-Trp receptor 2B
IGF2R	IGF2 Receptor
IL6R	IL6 receptor
KIT	Stem cell factor receptor
LHCGR	LH/CG receptor
MET	HGF receptor
NTRK1	Neurotrophic tyrosine kinase
PDGFRA	PDGF receptor alpha
PDGFRB	PDGF receptor beta
PLAUR	uPA receptor
PTCH	Patched homolog
PTCH2	Patched homolog 2
RET	RET oncogene
ROBO1	Roundabout, axon guidance receptor, homolog 1
ROBO2	Roundabout, axon guidance receptor, homolog 2
DNA repair proteins	
MLH1	MutL homolog 1
MSH2	MutS homolog 2
MSH3	MutS homolog 3
MSH6	MutS homolog 6
PMS1	PMS1 postmeiotic segregation increased 1
PMS2	PMS2 postmeiotic segregation increased 2
RAD51	RAD51 homolog (RecA homolog)
Apoptosis	
AKT1	AKT1 oncogene
APAF1	Apoptotic protease activating factor 1
BAK1	BCL2 antagonist killer-1
BAX	BCL2-associated X protein
BCL2	B-Cell lymphoma 2 oncogene
BCL2L1	Long isoform, (BCL-XL)
BCL2L1	Short isoform (BCL-XS)
BCLG	Apoptosis regulator BCL-G
BID	BH3-interacting domain death agonist
BIRC5	Survivin
CASP3	Caspase 3
CASP9	Caspase 9
HCS	Cytochrome C
PDCD8	Apoptosis-inducing factor (AIF)
SMAC	Diablo/Smac protein
Angiogenesis	
ANGPT1	Angiopoietin 1
ANGPT2	Angiopoietin 2
EFNB2	Ephrin-B2
EPHA3	Ephrin receptor EphA3
F2R	Thrombin receptor (TR); PAR1
F3	Tissue factor (TF)
FLT1	VEGF receptor 1 (VEGFR1)
FLT4	VEGF receptor 3 (VEGFR3)
HIF1A	Hypoxia-inducible factor 1 alpha
KDR	VEGF receptor 2 (VEGFR2)
MDK	Midkine (NEGF2)
NRP1	Neuropilin 1
PROK1	Prokineticin 1 (EG-VRGF)
SERPINB5	Serin proteinase inhibitor (Maspin)
TEK	TEK tyrosine kinase (TIE2)
THBD	Thrombomodulin
VEGF	VEGF-A
VEGF165	VEGF-A isoform
VEGFB	VEGF-B
VEGFC	VEGF-C
VEGFD	VEGF-D
XLKD1	Lymph. vessel endoth receptor1 (LYVE1)

Various (metabolic enzymes…)	
A2M	Alpha-2-macroglobulin
ABCB1	ATP-binding cassette (MDR1)
ACPP	Acid phosphatase, prostate
ANPEP	Alanyl aminopeptidase
APOA1	Apolipoprotein A-1
AZGP1	Alpha-2-glycoprotein 1
BR3	Carboxylesterase 3
CHGA	Chromogranin A
CYP1B1	Cytochrome P450, subfamily I, polypeptide 1
CYP3A4	Cytochrome P450, subfamily IIIA, polypeptide 4
CYP19	Cytochrome P450, subfamily XIX (Aromatase)
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3, alpha
DNMT3B	DNA methyltransferase 3, beta
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
EIF3S3	Eukaryotic translation initiation factor 3, subunit 3
EPHX1	Epoxide hydrolase 1 microsomal
ERVWE1	Endogenous retroviral family W
FUT1	Fucosyl transferase 1
GSTP1	Glutathione S-transferase pi
HDAC1	Histone deacetylase 1
HLA-C	MHC, class I, C
HP	Haptoglobin
HPN	Hepsin
KLK3	Kallikrein 3, (prostate specific antigen)
KLK4	Kallikrein 4 (prostase)
MECP2	Methyl CpG binding protein 2
NOS1	NNOS, neuronal nitric oxide synthase 1
NOS2A	iNOS, inducible hepatocyte NOS2A
NOS3	ENOS, NOS3 (endothelial)
SCGB1A1	Secretoglobin, family 1A, member 1 (Uteroglobin)
SCGB1D2	Secretoglobin, family 1D, member 2
SRD5A2	Steroid-5-alpha-reductase
SIAT1	Sialyl transferase 1
SOD1	Superoxyde desmutase 1
SOD2	Superoxyde desmutase 2
PACE	Paired basic amino acid cleaving enzyme (Furin)
PCA3	Prostate cancer antigen 3
STEAP	Six transmembrane epithelial antigen of the prostate
TERT	Telomere reverse transcriptase
TES	Testis derived transcript
TYMS	Thymidylate synthetase

Fig. 1 Continued.

Table 1 Clinical and histopathological features for 14 patients who underwent a radical prostatectomy for clinically prostate tumor

	Age[a]	PSA[a]	Pathological stage	Gleason score	Relapse[b]
T1	72	7.5	pT3 N0 M0	7	Yes
T5	65	19.7	pT2 N0 M0	6	Yes
T6	65	26.0	pT2 N0 M0	7	Yes
T8	73	10.5	pT2 N0 M0	7	No
T9	68	7.0	pT2 N0 M0	7	No
T20	69	5.2	pT2 N0 M0	7	No
T22	50	2.0	pT3 N0 M0	8	No
T25	60	8.0	pT3 N0 M0	7	Yes
T26	57	18.1	pT2 N0 M0	5	No
T41	54	10.0	pT2 N0 M0	7	Yes
T43	64	17.5	pT2 N0 M0	6	No
T46	67	8.8	pT3 N0 M0	5	No
T47	55	9.3	pT3 N0 M0	5	Yes
T49	71	8.2	pT3 N0 M0	7	Yes

[a] At time of diagnosis.
[b] At least 2 years of follow-up.

extraction and additional examination. This preselected tumor specimen was then sliced in the laboratory and again examined histologically. Samples were considered suitable for molecular studies when all examined epithelial cells were neoplastic. Malignant areas were carefully dissected with a scalpel, yielding a homogeneous cell population and avoiding dilution of tumor-specific genetic changes by nucleic acids from normal and reactive cells present in the same specimen. The histological diagnosis and clinical stage (based on the Tumor-Node-Metastasis system and Gleason score) were determined after surgery. Eight of the 14 selected tumors were stage pT2, and six were stage pT3. The Gleason score of the tumor area selected for RNA extraction was confirmed histologically by an experienced pathologist in the laboratory. The Gleason scores were 5–6 in five cases, 7 in eight cases, and 8 in one case.

With 2 years of follow-up, 7 of the 14 patients (three pT2 and four pT3) had progressed (as defined by two successive PSA values exceeding 0.2 ng/ml), whereas the other 7 patients (5 pT2 and 2 pT3) had not progressed.

The clinical characteristics of the 14 patients are shown in Table 1. Specimens of normal prostate tissue were obtained from 7 of these 14 patients who underwent radical prostatectomy and were used to determine basal target-gene mRNA expression. Normal-looking areas of each surgical specimen were examined histologically to confirm the absence of cancer cells and benign hyperplasia.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from tissue specimens by using the acid-phenol guanidinium method (5). The quality of RNA extracts was determined by electrophoresis through agarose gels, staining with ethidium bromide, and visualization of the 18S and 28S RNA bands under UV light. cDNA was synthesized as described previously (6).

Real-Time RT-PCR

Theoretical Basis. Quantitative values are obtained from the Ct number at which the increase in signal associated with exponential growth of PCR products starts to be detected (using PE Biosystems analysis software, according to the manufacturer's manual).

The precise amount of total RNA added to each reaction mix (based on absorbance) and its quality (*i.e.*, lack of extensive degradation) are both difficult to assess. We, therefore, also quantified *PPIA* (the peptidylprolyl isomerase A gene encoding cyclophilin A) and *RPLP0* (encoding the human acidic ribosomal phosphoprotein P0) transcripts as endogenous controls. *RPLP0* is also known as 36B4 and is widely used as an endogenous control for Northern blot analysis.

Results, expressed as N-fold differences in target gene expression relative to *PPIA* (or *RPLP0*) expression and termed "N*target*," were determined by the formula: N*target* = $2^{\Delta Ctsample}$, where the ΔCt value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the *PPIA* (or *RPLP0*) gene.

The N*target* values of the prostate samples were subsequently normalized such that the mean of the seven normal prostate N*target* values was 1.

Primers and PCR Consumables. Primers for *PPIA*, *RPLP0*, and the 291 target genes were chosen with the assistance of the Oligo 4.0 program (National Biosciences, Plymouth, MN). We performed Basic Local Alignment Search Tool (7) searches against dbEST, htgs, and nr (the nonredundant set of the GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan database sequences) to confirm the total gene specificity of the nucleotide sequences chosen as primers and the absence of DNA polymorphisms. In particular, the primer pairs were selected to be unique relative to the sequences of closely related family member genes or corresponding retropseudogenes. Primer sets were also tested by PCR to ensure they yielded a single band on agarose gel, and PCR products were purified and sequenced to confirm primer specificity. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or in a different exon (except for intronless genes). A primer pair lying in intron 12 of the albumin gene was used to check the DNA-free status of RNA samples (data not shown).

Moreover, genomic DNA was used as a template to confirm the RNA specificity of the primer set in each experiment.

For each set of primers, a no-template control and a no-reverse transcriptase control (reverse transcriptase-negative) assay, which produced negligible signals (usually Ct $>$ 35), were used to confirm the absence of primer-dimer formation and genomic DNA contamination.

Standard Curve Method. A relative kinetic method was applied using standard curve. The latter was constructed with 4-fold serial dilutions of cDNA from a pool of normal prostate tissues. Standard curves were produced for the 291 target genes, *PPIA*, and *RPLP0*.

PCR Amplification. All PCR reactions were performed using an ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems) and the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 45 cycles at 95°C for 15 s and 65°C for 1 min.

Inclusion Criteria for Target Gene Assay. In a first step, prostate tumor mRNAs were mixed to obtain two prostate

tumor pools [one representative of patients without relapse 2 years after radical surgery ($n = 7$) and one with relapse ($n = 7$)] and a pool of paired normal prostate specimens.

Tissue samples were considered suitable when *PPIA* Ct values were between 17 and 19, reflecting an appropriate starting amount and quality of total RNA.

The mean Ct values of the prostate samples included in the pools were 18.17 ± 0.29 (patients without relapse), 18.00 ± 0.47 (patients with relapse), and 18.22 ± 1.45 (normal prostate samples).

Statistical Analysis

The distributions of mRNA levels were characterized on the basis of their medians and ranges. Relationships between the target genes and clinical and histological parameters were tested with the nonparametric Mann-Whitney *U* test. Differences were considered to be significant at confidence levels greater than 95% ($P < 0.05$).

To visualize the ability of a given molecular marker to discriminate two populations (in the absence of an arbitrary cutoff value), we summarized the data in a ROC curve (8). This curve plots sensitivity (true positives) on the Y axis against one specificity (false positives) on the X axis, considering each value as a possible cutoff. The AUC were calculated as a single measure for the discriminatory capacity of each molecular marker. When a molecular marker has no discriminatory value, the ROC curve lies close to the diagonal and the AUC is close to 0.5. When a molecular marker has strong discriminatory value, the ROC curve moves to the top left or bottom right corner and the AUC approaches 1 or 0.

Hierarchical clustering was performed using GeneANOVA software (9). The data sets were rank-transformed before analysis by the WARD method.

RESULTS

Gene Expression Profile in Clinically Localized Prostate Tumors. mRNA levels of 11 (3.8%) of the 291 target genes were very weak in both prostate tumor pools and in the normal prostate pool, being detectable but not reliably quantifiable (Ct > 30) by means of real-time quantitative RT-PCR with fluorescence SYBR Green methodology.

mRNA levels of each of the 291 genes were determined in the two prostate tumor pools and were compared with the mRNA levels of each gene in the normal prostate pool. The 88 genes displaying markedly altered expression (at least a 2-fold difference between at least one prostate tumor pool and the pool of normal prostate tissues) were selected for additional analysis in the 14 individual prostate tumors and seven paired normal prostate specimens.

Mean expression values obtained with the individual prostate tumors were very similar to those obtained with the corresponding pooled samples (data not shown). Median mRNA levels of 46 (52.3%) of these 88 genes differed significantly between the 14 prostate tumors and the seven normal prostate samples ($P < 0.005$, Mann-Whitney *U* test; Table 2). Five genes showed significantly higher expression in the tumor specimens, and 41 showed significantly reduced expression.

With regard to the five genes with significantly increased expression, the ratio of the median value in the 14 prostate tumors and the median value in the seven normal prostate tissues ranged between 1.8 (*MMP9*) and 5.7 (*CDKN2A*). With regard to the 41 genes with significantly reduced expression, the ratio of the median value in the 14 prostate tumors and the median value in the seven normal prostate tissues ranged between 0.07 (*NRG1*) and 0.63 (*TYMS* and *UNC5C*).

The capacity of each of these 46 genes to discriminate between tumoral and normal prostate tissue was assessed by using the AUC-ROC method. The AUC values shown in Table 2 represent the capacity of each gene to discriminate between normal and tumoral prostate tissue. The N*target* values indicated in Table 2 (calculated as described in "Materials and Methods") were based on the amount of the target message relative to the *PPIA* endogenous control, to normalize the amount and quality of total RNA; similar results were obtained by using a second endogenous RNA control, the *RPLP0* gene (also known as 36B4; data not shown).

The only associations between clinical and pathological features (*e.g.*, age, serum PSA level at diagnosis, stage, and Gleason score) and the expression of individual target genes were between *ILK* and *F3* expression and the Gleason score ($P = 0.05$ and $P = 0.009$, respectively); increased *ILK* expression was associated with moderately or poorly differentiated tumors, and reduced *F3* expression was associated with poorly differentiated tumors.

Predictors of Clinical Outcome. With 2 years of follow-up, 7 of the 14 patients (three pT2 and four pT3) had progressed (as defined by two successive PSA values exceeding 0.2 ng/ml), whereas the other 7 patients (five pT2 and two pT3) had not progressed (Table 1).

The capacity of each gene to discriminate patients who relapsed from patients who did not relapse was estimated by the AUC-ROC method. By combining the two genes with the AUC-ROC values closest to 0 (*XLKD1/LYVE1* and *CGA*; 0.061 and 0.143, respectively) and the two genes with the AUC-ROC values closest to 1.0 (*F2R/PAR1* and *BCL-G*; 0.878 and 0.714, respectively), we obtained a four-gene model that discriminated perfectly between the seven patients with and the seven patients without relapse, independently of stage and grade. *XLKD1/LYVE1* and *CGA* expression was significantly lower ($P < 0.05$, Mann-Whitney *U* test) in patients who relapsed than in patients who did not relapse (median values: 0.06 *versus* 0.24 and 0.36 *versus* 3.55, respectively), whereas *BCL-G* and *F2R/PAR1* expression was significantly higher (1.04 *versus* 0.48 and 1.27 *versus* 0.58, respectively; data not shown).

The gene clusters are shown as dendograms (Fig. 2), in which line length and branching reflect the relatedness of the samples according to the expression of the four genes.

DISCUSSION

We studied the mRNA expression of 291 genes involved in pathways (*e.g.*, cell cycling, signal transduction, apoptosis, angiogenesis, and metastasis) that are known to be dysregulated in solid tumors, including CaP.

Eighty-eight genes showed at least a 2-fold difference in expression between pooled prostate tumors and pooled normal prostate tissues. The expression level of these 88 genes was then

Table 2 List of the significantly altered expressed genes in prostate tumors compared with normal prostate specimens

Genes	Normal prostate specimens ($n = 7$)[a]	Prostate tumors ($n = 14$)	Ratio	ROC-AUC
XDLD1 (*LYVE1*)[b]	0.93[c]	0.11[d]	0.116	0.000
ILK	0.97	0.46	0.474	0.009
DCC	0.97	0.12	0.122	0.013
NRG1	0.87	0.06	0.072	0.017
BMP7	1.02	0.35	0.344	0.024
NOS1	1	0.09	0.095	0.025
SRD5A2	1.12	0.16	0.144	0.032
SERPINB5 (Maspin)	1.04	0.11	0.103	0.037
FGR2IIIB	0.88	0.25	0.282	0.037
ITG3B	0.99	0.39	0.393	0.041
NTN1 (netrin-1)	0.95	0.25	0.267	0.045
GSTP1 (GSTπ)	1.04	0.35	0.336	0.049
ITGA5	0.83	0.24	0.285	0.053
GRO1	1.01	0.22	0.218	0.055
AREG	1.15	0.16	0.135	0.063
PROK1	1	0.12	0.124	0.067
SPARC-LIKE	0.98	0.6	0.611	0.070
IGB4	0.99	0.29	0.296	0.072
CALD1	1	0.39	0.395	0.072
PIA2	0.79	0.17	0.21	0.075
THBD (thrombomodulin)	0.91	0.28	0.309	0.077
ROBO 1	0.87	0.34	0.391	0.078
GJA1 (connexin 43)	1.02	0.49	0.48	0.100
MMP14	0.94	0.51	0.54	0.105
CD44	1.02	0.45	0.441	0.117
NOS2A	0.91	0.23	0.259	0.124
SIAT	0.93	0.38	0.41	0.130
SFN (Stratifin)	1.25	0.22	0.177	0.133
TYMS	0.86	0.55	0.632	0.148
CD13	0.65	0.06	0.087	0.149
CXR4	0.96	0.46	0.479	0.157
CYP1B1	0.97	0.49	0.505	0.158
GRP	0.96	0.17	0.177	0.165
CAV1	1.01	0.28	0.281	0.193
CHGA	0.78	0.12	0.154	0.200
RET	1.18	0.23	0.198	0.208
ROBO 2	0.87	0.47	0.540	0.212
UNC5C	0.84	0.53	0.631	0.221
CDKN2B (*p15*)	0.94	0.49	0.521	0.222
SLIT 3	0.84	0.48	0.571	0.255
EDNRB (*ETB*)	0.78	0.48	0.615	0.273
COL1A1	0.94	1.89	2.004	0.761
MMP9	1.08	1.96	1.812	0.785
PCA3 (*DD3*)	0.99	2.18	2.202	0.829
GRPR	1	3.14	3.143	0.885
CDKN2A (*p16*)	0.89	5.09	5.686	0.969

[a] Mann-Whitney *U* test.
[b] Locus Link (Usual name).
[c,d] Numbers represent N*target* (see "Materials and Methods") medians of normal prostate specimens and prostate tumors, respectively.

studied individually in 14 prostate tumors and seven normal prostate specimens. Analysis of the expression level of these 88 genes in each sample further allowed us to check for potential heterogeneity within each pool. Mean values calculated for individual prostate tumors were very close to the value obtained for the corresponding pooled samples (data not shown), indicating that the initial evaluation based on pooled samples was reliable. The expression of 5 of the 88 selected genes was significantly increased, whereas that of 41 genes was significantly decreased (Table 2). It is noteworthy that the proliferative marker *MKI67* had no discriminatory value, the ROC curves lying close to the diagonal and the AUC value being close to 0.5 (AUC-ROC, 0.479; data not shown), suggesting that the dysregulation of the 46 target genes in prostate tumors was not caused by the high proliferative activity of cancer cells.

The gene expression pattern in prostate tumors was related to: (*a*) complex physiological properties (*e.g.*, angiogenesis and invasion); (*b*) the activity of specific signaling pathways (*e.g.*, *DCC*, *NTN1*, and *ROBO*); and (*c*) cell specificity (*e.g.*, stroma-dependent expression of growth factors).

First, most of the dysregulated genes belonged to the ECM and ECM-remodeling categories and were involved in angiogenesis (*e.g.*, *XLKD1/LYVE1*, *PROK1*, *SERPINB5/Maspin*, and *THBD*). The MMP gene *MMP9* was overexpressed in the tu-

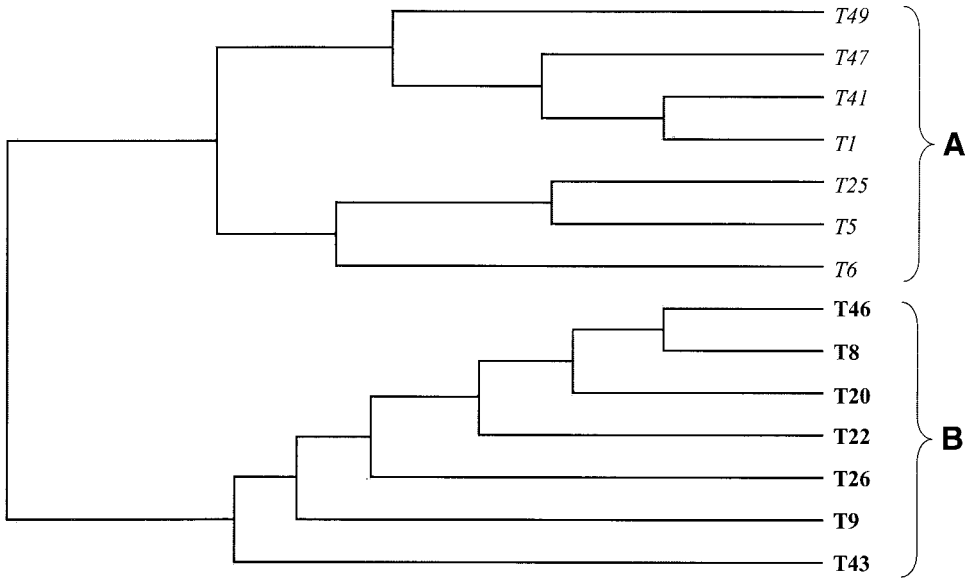

Fig. 2 Hierarchical clustering was applied to 14 clinically localized prostate tumors on the basis of expression data from four genes (*XLKD1/LYVE1*, *CGA*, *F2R/PAR1*, and *BCL-G*). These four genes were selected from a total of 291 genes by the AUC-ROC test described in "Materials and Methods." *A*, patients with relapse. *B*, patients who remained relapse free for at least 2 years after surgery.

mors, whereas all of the other genes (ECM, ECM-remodeling, and angiogenesis dysregulated genes: *MMP14*, *ITGA5*, *ITGB4*, *ITGB3*, *XLKD1/LYVE1*, *PROK1*, *SERPINB5/Maspin*, and *THBD*) were underexpressed. Except for *CDKN2A*, no genes involved in cell cycling, DNA synthesis, or gene transcription activities related to the highly proliferative status of cancer cells were dysregulated. Similarly, no genes involved in apoptotic pathways were significantly dysregulated. However, the anti-apoptotic gene *BIRC5/Survivin* was expressed about 1.6 times more strongly in the tumors than in the normal prostate tissues (data not shown).

Second, several key genes in axon guidance and cell migration were underexpressed in prostate tumors, including *NTN1*/netrin-1, the chemoattractant gene and its receptor *DCC* (deleted in colorectal cancer), and *SLIT3* and its *ROBO* (Roundabout) receptors. These genes might, therefore, be involved in the chemotactic behavior of CaP cells.

Finally, most of the growth factor genes dysregulated in prostate tumors were down-regulated (*AREG*, *NRG1*, *BMP7*, *GRO1*, and so forth), possibly because of the stroma-dependent nature of their expression.

Some of the genes found to be dysregulated here have already been forwarded as cancer biomarkers or mediators of prostate carcinogenesis. For example, *PCA3* (also known as *DD3*) has already been shown to be overexpressed in prostate tumors (10) and is a very sensitive and specific marker of this malignancy (11); *GRPR* (gastrin-releasing peptide receptor), which has high affinity for gastrin-releasing peptide and bombesin, has also been shown to be overexpressed (12), and therapeutic approaches based on bombesin receptor antagonists and cytotoxic bombesin analogues have been considered in CaP (13). We also found a 9.7-fold decrease in the expression of *SERPINB5/maspin*, in keeping with the role of the corresponding protein as an angiogenesis inhibitor (14) that acts at the cell membrane to inhibit the invasiveness and motility of prostatic cancer cells (15).

We identified some other genes or their products as potential therapeutic targets in CaP.

MMP9 was overexpressed, and synthetic inhibitors of MMPs have therapeutic efficacy in various cancers. Recently, a novel inhibitor, Ro 28–2653, with high selectivity for *MMP9*, showed promise in patients with CaP (16).

CDKN2A exon 1α (p16) transcripts were overexpressed in prostate tumors; indeed, 8 (57%) of 14 prostate tumors displayed increased expression of p16*CDKN2A*, varying from 2- to 10-fold higher than normal prostate tissues. In contrast, a decrease in p16*CDKN2A* expression was found in three prostate tumors (data not shown). The product of this gene is a cyclin-dependent kinase inhibitor that functions as a cell growth regulator controlling cell cycle G_1 progression through engagement of the Rb-cdk4/6-cyclin D pathway (17). p16*CDKN2A* overexpression has already been described in solid tumors including CaP (18). An altered *RB1* axis could trigger p16*CDKN2A* overexpression in certain systems. However, we found no association, at the mRNA level, between p16*CDKN2A* and *RB1* or other cell cycle regulator expression.

It is of note that the $p14^{ARF}$ product encoded by this gene, through an alternate open reading frame, was expressed 1.6 times more strongly in the tumors than in the normal prostate tissues (data not shown).

Other interesting candidate targets are key genes in axon guidance and cell migration in the central nervous system, such as the secreted proteins netrin-1 and slit3.

We then analyzed the prostate tumor gene expression signature according to tumor recurrence. Recurrence is a major problem in this setting, because 30% of men undergoing radical prostatectomy for localized tumors eventually relapse, and none of the current indicators of progression (stage and grade at diagnosis) can predict outcome (Table 1). There is, thus, an urgent need for robust prognostic markers capable of identifying patients at risk of relapse after radical surgery.

In this study, the patients were all assessable with respect

to recurrence after surgery; seven patients relapsed (defined as two successive PSA values >0.2 ng/ml), and seven remained relapse free for at least 2 years. We used the AUC-ROC test to assess the prognostic value of each gene in terms of relapse. A four-gene model based on the two genes with the AUC values closest to 0 (*XLKD1/LYVE1* and *CGA*) and the two genes with the AUC values closest to 1 (*F2R/PAR1* and *BCL-G*) perfectly discriminated between the seven patients who relapsed and the seven patients who did not relapse after 2 years of follow-up (Fig. 2). *XLKD1/LYVE1* and *F2R/PAR1* encode angiogenic factors *BCL-G*, a proapoptotic factor, and *CGA*, a growth factor.

CGA is the α-subunit of the human glycoprotein hormone chorionic gonadotropin. *CGA* was overexpressed in patients who did not relapse, in keeping with our previous work showing that *CGA* is a specific *ER*α -responsive gene in CaP and that its overexpression may be associated with a good prognosis in CaP (19) as in breast cancer (20).

XLKD1/LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium (21). The functional role of *XLKD1/LYVE-1* in lymphatic vessels, and its use as a marker of tumor lymphangiogenesis, are important areas of investigation. In our study, the association between low *XLKD1/LYVE-1* expression and poor outcome is in keeping with the known function of this gene; indeed, *XLKD1/LYVE-1*-positive structures have been observed in lung tumor margins but not within the mass of such tumors (22). Moreover, negative intratumoral XLKD1/LYVE-1 staining does not rule out metastasis (23), suggesting that functional lymphatics in the tumor margins are sufficient for lymphatic metastasis (22).

We also found that increased *F2R/PAR1* expression was associated with tumor recurrence. This coagulation factor II (thrombin) receptor is involved in regulating the thrombotic response. Thrombin promotes angiogenesis by enhancing vascular endothelial growth factor synthesis and inducing its secretion and has already been implicated in prostate tumorigenesis and metastasis (24).

Last, overexpression of *BCL-G*, encoding a BCL2 family protein, has been shown to induce apoptosis in COS-7 cells (25). However, little is know about the expression of this gene in tumor samples, and *BCL-G* has not been linked previously to clinical outcome.

Our results indicate that although *F2R/PAR1* and *BCL-G* overexpression are associated with poor outcome, optimal outcome prediction is obtained by their combination with genes, such as *CGA*, that are associated with good outcome. The resulting four-gene model was perfectly predictive of tumor recurrence, independently of tumor stage and grade. It is noteworthy that *F3* and *ILK*, the expression of which correlates with the Gleason score, a current prognostic marker, were excluded from this model.

In conclusion, our data identify potential new therapeutic targets in CaP and show that gene expression profiling can be used as a predictor of outcome. Routine clinical use of genomics-based outcome predictors must await confirmation in larger, independent data sets.

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Clin Cancer Res 2003;9:5477-5485.