Supporting Materials and Methods

Tissue Acquisition. All materials were acquired and used in conformity with Institutional Review Board-approved protocols at the University of Washington and Oregon Health & Science University. Two types of tissue were used for this study: fresh, nonfixed tissue as a source of RNA for laser microdissection and formalin-fixed, paraffin-embedded tissue for immunohistochemistry studies. The nonfixed tissue consisted of (*i*) frozen tissue blocks from 29 radical prostatectomies accessioned and selected to represent a range of Gleason grades and the absence of any treatment before prostatectomy and (*ii*) an independent sample set of frozen tissue blocks containing prostate needle core biopsies from 30 subjects. To minimize RNA degradation in the radical prostatectomy tissue, 3-mm-thick transverse sections were made upon receipt of the specimen, and tissue blocks from the posterior aspect of each transverse section were placed immediately in optimal cutting temperature medium (OCT) and immersed in isopentane that had been precooled in liquid nitrogen. The frozen blocks were stored at –80°C until use. Fixed tissue samples consisted of two types, blocks corresponding to the fresh tissue samples that were used for the laser microdissection preparations and tissue microarrays (TMAs), which are described below.

Laser-Capture Microdissection and RNA Preparation. Frozen sections (8 μm) were cut from OCT blocks and immediately fixed in cold 95% ethanol. After brief (5–10 seconds) staining with hematoxylin using the HistoGene Staining Solution (Arcturus Engineering, Mountain View, CA) the sections were dehydrated in 100% ethanol, followed by xylenes (per the manufacturer's protocol). Epithelial cells (≈5,000) from both histologically benign glands and cancer glands were separately laser-capture microdissected (LCM) by using the Arcturus PixCell II instrument. Only one Gleason pattern was included in each laser-captured cancer sample. A total of 32 different Gleason patterns were captured from the 29 radical prostatectomy samples: 12 Gleason pattern 3, 12 Gleason pattern 4, and 8 Gleason pattern 5 samples. A total of 30 Gleason patterns were captured from the 30 needle core biopsy samples, with some samples composed of combinations of Gleason patterns. Matched benign epithelium was captured

for each cancer sample for a total of 121 samples. Digital photos were taken of tissue sections before, during, and after LCM and assessed independently by two investigators to confirm the Gleason patterns of the laser-captured cells.

After LCM, captured cells were lysed in RNA extraction buffer (Arcturus). RNA was isolated by using the PicoPure RNA Isolation kit (Arcturus) and the samples were DNase treated by using RNase-Free DNase (Qiagen). Each slide capture session lasted no longer than 20 min to minimize RNA degradation. The laser-capture settings were 55-mW beam, 1.5-ms pulse, and 15-μm spot size. Subsequently, RNA was amplified through two rounds of linear amplification using the MessageAmp aRNA kit (Ambion). Sample quality and quantification was assessed by agarose gel electrophoresis and absorbance at A260.

Microarray Construction and Hybridization. Prostate Expression Database (PEDB) cDNA microarrays were prepared on polylysine-coated glass microscope slides (Gold Seal; Becton Dickinson) by using a robotic spotting tool (GeneMachine OmniGrid 100) as described in ref. 1. The PEDB array consists of ≈7,000 human prostate-derived cDNA clones identified from the PEDB, which is a sequence repository of human prostate EST data available to the public (www.pedb.org) (2) plus ≈500 human cDNAs chosen from the Research Genetics sequence-verified set of IMAGE clones (http://www.resgen.com/products/SVMcDNA.php3). Additional control and reference cDNAs were included, making a total of 7,700 unique cDNAs represented on the PEDB array. All clones were replicate-spotted, for a total of 15,488 spots per array.

The protocol used for indirect labeling of cDNAs was a modification of a protocol described elsewhere (http://cmgm.stanford.edu/pbrown/protocols/aadUTPCouplingProcedure.htm). In brief, cDNA probes were made from 2 μg of amplified RNA in a reaction volume of 30 μl containing 5 μg of random hexamer primers, 0.2 mM 5-(3-aminoallyl)-2_-deoxyuridine-5_-triphosphate (amino acid-dUTP; Sigma-Aldrich), 0.3 mM dTTP, 0.5 mM each dATP, dCTP, and dGTP, and 380 units of Superscript II reverse transcriptase (Invitrogen)

incubated at 42°C for 120 min. After RNA hydrolysis, purified cDNA was combined with either Cy3 or Cy5 monoreactive fluors (Amersham Pharmacia) that covalently couple to the cDNA-incorporated aminoallyl linker in the presence of 50 mM NaHCO3 (pH 9.0). Samples were randomly labeled with either Cy3 or Cy5 dye to account for dye bias. The coupling reaction was quenched with hydroxylamine. Patient-matched normal and cancer probes were combined, filtered, and competitively hybridized to microarrays under a coverslip for 14 h at 63°C. Slides were washed sequentially with 1× saline sodium citrate (SSC)/0.03% SDS, 1× SSC, 0.2× SSC, and 0.05× SSC and spun dry.

Microarray Data Acquisition and Analysis. Fluorescent array images were collected for both Cy3 and Cy5 emissions by using a GenePix 4000B fluorescent scanner (Axon Instruments, Foster City, CA). The image-intensity data were gridded and extracted by using GENEPIX PRO 4.1 software. The following processing procedures were applied separately to the radical prostatectomy samples and the prostate needle core biopsy samples.

Log ratios. For each spot and in each channel (Cy3 and Cy5), the median background intensity was subtracted from the median foreground intensity. Log ratios of cancer expression to benign expression were created by first dividing the background-subtracted intensities (CaP/Benign) and then taking the log base 2. If the median background intensity was greater than the median foreground intensity, the spot was considered missing.

Removal of control genes. The array contained ≈400 clones used only for quality control purposes (i.e., yeast sequences or blank spots). These clones were removed from the data set.

Lowess normalization. For each array, the log-ratio data were centered by using a print-tip-specific Lowess curve (Yang, Y. H., Dudoit, S., Luu, P. & Speed, T. P. (2001) *Normalization for cDNA Microarray Data*. (SPIE BiOS 2001, San Jose, CA). This curve was fit to the log-intensity versus log-ratio plot by using the neighboring 20.0% of the

data to calculate the fit at each spot. The Lowess fit at each point was subtracted from the observed log ratio for that spot, resulting in a normalized log ratio.

Assessing spot quality. Spots of poor quality, as determined by both visual inspection and GENEPIX PRO 4.1 quality flags were considered missing. In addition, spots with background-subtracted intensity levels <300 were considered missing because of poorly hybridized cDNAs. Clones that were missing on >20% of arrays were removed from the analysis.

Imputation. Missing values were imputed by using *k*-nearest-neighbors imputation (k = 10) (2). The data set was split by Gleason pattern (Grades 3, 4, and 5), and imputation was performed separately for each pattern.

Average replicated clones. Log ratios from the replicated cDNA spots on each PEDB chip were averaged after normalization and imputation. These average expression values were used for comparative analysis.

Filtering. The radical prostatectomy data were filtered to include (*i*) clones whose average expression (log ratio) was nonmissing in at least half of one of the Gleason pattern groups and (*ii*) clones whose expression ratios (benign versus cancer) were 1.5-fold or greater in at least half of one of the Gleason groups. The prostate needle core biopsy data were not filtered in this manner, because the clone set for analysis was chosen by using the prostatectomy data.

Statistical analysis. To compare the overall expression patterns of all radical prostatectomy cancer samples to their patient-matched normal samples, the filtered log-ratio measurements were analyzed by using the Significance Analysis of Microarrays (SAM) procedure (3) (www-stat.stanford.edu/~tibs/SAM). This procedure calculates an attenuated *t* test statistic for each gene, where a small value is added to the standard deviation to help reduce the occurrence of statistically significant but biologically unimportant genes, i.e., statistically significant genes with a very low level of differential

expression. Statistical significance is corrected for multiple comparisons by using the false discovery rate. In this analysis, a one-sample *t* test was used to determine which genes were significantly differentially expressed between cancer samples and their patient-matched normal samples. We call the set of significant genes the expression profile associated with prostate cancer.

To identify gene expression alterations associated with specific Gleason patterns, we used Prediction Analysis for Microarrays (PAM) (4), a supervised classification method. This method is a modification of standard nearest-centroid or nearest-neighbor classification. For each class, the average expression across samples (class centroid) is calculated, and new samples are classified based on their similarity to, or distance from, the class centroids. PAM is a similar method of analysis, with the difference that only genes that most differentiate the individual classes are retained in the analysis. A thresholding parameter, δ, determines how many genes are retained; the value of δ is chosen by using leave-out-one cross-validation analysis. Full technical details are provided on the PAM website (www-stat.stanford.edu/~tibs/PAM).

We divided the radical prostatectomy samples into two classes (Gleason pattern 3 and Gleason pattern 4 or 5) and applied PAM to identify several small gene cohorts that classified the samples with low error rates under leave-one-out cross validation. To evaluate the predictive properties of these gene sets, we classified an independent sample set of Gleason pattern cancers, the prostate needle core biopsy samples.

An additional visual assessment of the degree to which our gene model partitioned the radical prostatectomy samples by Gleason pattern was undertaken by using principal components analysis (PCA) of the samples (5). PCA is a linear transformation of high-dimensional data, in this case, gene expression values, which reduces the dimensionality while preserving between-sample variability. Using PCA, we were able to visualize in two dimensions the gene-expression profile of each sample and the relationship between gene-expression profiles.

Quantitative RT-PCR (qRT-PCRs). cDNA was generated from 1 μg of aRNA by using 2 μg of random hexamers for priming reverse transcription by SuperScript II (200 units per reaction; Invitrogen). Primers and salts were removed by using a PCR product isolation kit (Qiagen). qRT-PCRs were done in triplicate, using ≈5 ng of cDNA, 0.2 μM each primer, and SYBR green PCR master mix (Applied Biosystems) in a 20-μl reaction volume. Reactions were carried out and analyzed by using an Applied Biosystems 7900 sequence detector. Samples were normalized to the cycle threshold value obtained during the exponential amplification of RPL13A. The expression level of HSD17B4 was calculated. Values were reported as the ratio of gene expression in neoplastic to normal epithelium. Control reactions with RNA or water as template did not produce significant amplification products. Amplification of a single PCR product per reaction was monitored by generation of a single dissociation curve. The sequences of primers used in our study were: RPL13A forward, 5-CCTGGAGGAGAAGAGGAAAGAGA-3; RPL13A reverse, 5-TTGAGGACCTCTGTGTATTTGTCAA-3; HSD17B4 forward, 5-GACTACGCCAAGCTCTGAAGG-3; and HSD17B4 reverse, 5-AAGCAATTTC CCCTGCATCTT-3.

TMA. Eight TMAs were used for these studies. All samples in all arrays were provided in duplicate. Two arrays of predominantly primary prostate cancers (of 159 samples and 234 samples, respectively) have been described (6). Six arrays represented a range of Gleason grades, a mix of prostate cancer tissue of different biologic states [normal, atrophy, benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), primary prostate carcinoma, and metastatic prostate carcinoma] and a mix of different normal and neoplastic tissues. Altogether, 469 unique samples of normal prostate glands and 889 unique samples of primary prostate carcinoma (572 Gleason pattern 3, 276 Gleason pattern 4, and 41 Gleason pattern 5) were used for monoamine oxidase A (MAOA) immunostaining, and 131 unique samples of benign prostate glands and 306 unique samples of primary prostate carcinoma (211 Gleason pattern 3, 77 Gleason pattern 4, and 18 Gleason pattern 5) were used for defender against cell death 1 (DAD1) immunostaining.

Immunohistochemistry. MAOA:Rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 458–527, mapping at the carboxy terminus of human-origin MAOA was obtained from Santa Cruz Biotechnology (product number sc-20156) and used at a dilution of 1:2,000. DAD1:rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 1–113, representing full length of DAD1 of human origin was obtained from Santa Cruz Biotechnology (product number sc-25557) and used at a dilution of 1:20.The titer of each antibody was selected to minimize nonspecific staining. Specificity of labeling was confirmed by both omission of the primary antibody and by immunostaining the sections with a primary antibody against an irrelevant antigen. Immunolocalization was done by using a three-step avidin–biotin–peroxidase method. In brief, deparaffinized sections were rehydrated in PBS and subjected to antigen retrieval by using a microwave (15 min in citrate buffer solution for MAOA and 20 min in the same buffer for DAD1). Sections were then incubated sequentially in solutions of 5% albumin in PBS, 10% hydrogen peroxide in water, primary antibody, secondary antibody [biotinylated anti-rabbit IgG (BA-1000; Vector Laboratories)] and avidin–biotin–peroxidase solution (Vector Laboratories), with interval washes in PBS. Reaction product was detected by incubating the sections in an aqueous solution of 0.05% diaminobenzidine and 0.3% hydrogen peroxide. The sections were counterstained with hematoxylin.

Evaluation of Immunohistochemical Stains. Immunohistochemical stains were evaluated by using the following categorical compositional scale: 0, no expression; 1, ≤5% of the cells express the antigen; 2, 5–20% of the cells express the antigen; and 3, 20–100% of cells express the antigen. The following cell types were evaluated: secretory and basal epithelial, high-grade PIN, and Gleason pattern 3, Gleason pattern 4, and Gleason pattern 5 tumor cells. If a section had several Gleason patterns, each pattern was scored.

To test for differences in the staining intensity of different cell types, we used the proportional-odds model (McCullagh, P. & Nelder, J. A. (1989) *Generalized Linear*

Models (Chapman and Hall, London). The proportional odds model has the following form:

$\log\left(\frac{\gamma_j(\vec{x})}{1-\gamma_j(\vec{x})}\right) = \alpha_j + \vec{\beta}^T\vec{x}$, $j = 1$, where $\gamma_j(\vec{x})$ is the probability that a given section has intensity j or less, given the covariate vector $\vec{x}$. We included the covariates Gleason grade and tissue source (multiple TMAs were combined) in our model. The model was fit in SAS (SAS Institute, Cary, NC) by using proc gen mod and implementing a Generalized Estimating Equations approach to account for multiple sections from the same patient. From the fitted model parameters, $\vec{\beta}^T$, we estimated the odds of observing intensity $<j$ at $x = \vec{x}_1$ and $x = \vec{x}_2$, namely, $\left(\frac{\gamma_j(\vec{x}_1)/(1-\gamma_j(\vec{x}_1))}{\gamma_j(\vec{x}_2)/1-\gamma_j(\vec{x}_2)}\right) = \exp(-\vec{\beta}^T(\vec{x}_1 - \vec{x}_2))$.

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