Identifying distinct classes of bladder carcinoma using microarrays

Lars Dyrskjøt[1], Thomas Thykjaer[1,2], Mogens Kruhøffer[3], Jens Ledet Jensen[4], Niels Marcussen[5], Stephen Hamilton-Dutoit[5], Hans Wolf[3] & Torben F. Ørntoft[1]

Published online 9 December 2002; doi:10.1038/ng1061

Bladder cancer is a common malignant disease characterized by frequent recurrences[1,2]. The stage of disease at diagnosis and the presence of surrounding carcinoma *in situ* are important in determining the disease course of an affected individual[3]. Despite considerable effort, no accepted immunohistological or molecular markers have been identified to define clinically relevant subsets of bladder cancer. Here we report the identification of clinically relevant subclasses of bladder carcinoma using expression microarray analysis of 40 well characterized bladder tumors. Hierarchical cluster analysis identified three major stages, Ta, T1 and T2-4, with the Ta tumors further classified into subgroups. We built a 32-gene molecular classifier using a cross-validation approach that was able to classify benign and muscle-invasive tumors with close correlation to pathological staging in an independent test set of 68 tumors. The classifier provided new predictive information on disease progression in Ta tumors compared with conventional staging ($P < 0.005$). To delineate non-recurring Ta tumors from frequently recurring Ta tumors, we analyzed expression patterns in 31 tumors by applying a supervised learning classification methodology, which classified 75% of the samples correctly ($P < 0.006$). Furthermore, gene expression profiles characterizing each stage and subtype identified their biological properties, producing new potential targets for therapy.

Parallel gene-expression monitoring is a powerful tool for analyzing relationships between tumors, discovering new tumor subgroups, assigning tumors to pre-defined classes, identifying co-regulated or tumor stage–specific genes and predicting disease outcome[4–17]. In a recent study of bladder cancer, we identified functional groups of genes whose co-regulation formed the basis for separating bladder tumors into superficial and muscle-invasive tumors[18]. Here, we used microarrays to analyze gene expression and to predict tumor classes in 40 bladder tumors from a clinical specimen bank holding more than 35,000 samples selected on the basis of disease course, stage, grade, concomitant carcinoma *in situ* (CIS) and recurrence frequency (number of new tumors per year; Table 1 and Web Table A online). We labeled RNA from tumors and from four normal tissue samples and hybridized it to Affymetrix oligonucleotide microarrays. We then subjected the 1,767 genes (26%) that were expressed at different levels in tumor tissue versus normal urothelium to a two-way hierarchical cluster analysis. This separated all 40 tumors according to conventional pathological stages and grades with only a few exceptions (Fig. 1*a*). The distinct separation of the tumor groups according to stage with practically no overlap between groups was also shown by multidimensional scaling analysis (Fig. 1*b*).

To reduce the number of genes needed for class prediction, we identified the 88 genes that varied most between tumor samples (s.d. ≥ 4) and that were considered to be cancer-related by the Cancer Genome Anatomy Project (CGAP; at the US National Cancer Institute). Hierarchical clustering using only these genes was almost identical to that using the 1,767 genes (Fig. 1*c*), indicating that the tumor clustering does not simply reflect larger amounts of stromal components in the invasive tumor samples. Many cell types are present in the biopsy samples, however, and the specific origin of the transcript cannot be determined.

The clustering of the 1,767 genes identified several characteristic profiles that differed between the tumor groups (Fig. 1*d*). Cluster A was highly expressed in all the Ta grade 3 tumors (Fig. 2) and contains genes encoding eight transcription factors and other nuclear genes related to transcriptional activity. Cluster C contains genes that were upregulated in Ta grade 3 tumors with high recurrence rate and CIS and in T2+ and some T1 tumors. This cluster showed a tight co-regulation of genes related to cell-cycle control and mitosis (Fig. 2), and thus may be associated with increased cellular proliferation and may serve as new targets for small-molecule therapy[19]. Cluster F showed a tight cluster of genes related to keratinization (Fig. 2). Two tumors (875-1 and 1178-1) highly expressed these genes, and a re-evaluation of the pathology slides indicated that these were the only two samples to show squamous metaplasia, a characteristic not infrequently seen in invasive bladder tumors by light microscopy. Cluster G contains genes related to angiogenesis and connective tissue that were upregulated in T2+ tumors and in the Ta grade 3 tumors with CIS that clustered in the invasive branch (Fig. 2). Increased transcription of these genes may indicate a profound remodeling of the stroma, and thus they may also serve as new targets for drug therapy[20]. Notably, these genes most clearly separated the Ta grade 3 tumors surrounded by CIS from all other Ta grade 3 tumors. This

[1]*Molecular Diagnostic Laboratory, Department of Clinical Biochemistry, Aarhus University Hospital, Skejby, DK-8200 Aarhus N, Denmark.* [2]*Aros Applied Biotechnology, SciencePark Aarhus, Gustav Wiedsvej, Aarhus C, Denmark.* [3]*Department of Urology, Aarhus University Hospital, Skejby, Aarhus N, Denmark.* [4]*Department of Theoretical Statistics, Department of Mathematical Sciences, Ny Munkegade, Aarhus C, Denmark.* [5]*University Institute of Pathology, Aarhus University Hospital, Aarhus C, Denmark. Correspondence should be addressed to T.F.Ø. (e-mail: orntoft@kba.sks.au.dk).*

indicates that analysis of stroma-remodeling genes in Ta tumors could replace the present invasive procedure for diagnosing CIS.

Clusters B, D, E, H, I and J contain genes related to nuclear proteins, cell adhesion, growth factors, stromal proteins, immune system and proteases, respectively (see Web Fig. A online). A summary of the stage-related gene expression is shown in Table 2. The gene clusters are based on tumor biopsy samples, which contain contributions from many cell types as well as malignant cells.

An objective prediction of classes of bladder tumors using a limited set of genes could be of potential clinical use. We therefore built a maximum-likelihood classifier using only those tumors (35 of 40) that showed a group-specific expression pattern (see Web Fig. B online). We evaluated the classifier using a 'leave one out' cross-validation scheme[11,12] and selected for classification those predictive genes that showed the largest possible separation of the three groups. Each tumor was classified according to its proximity to the mean of the three groups (Fig. 3*a*). We tested the classifier's performance using 1–200 genes in cross-validation loops, and obtained the best correlation to pathologic staging by using a 38-gene cross-validation scheme (see Web Fig. C online). We selected 32 genes that were used in at least 75% of the cross-validations (27 times) to constitute our final classifier model (see Web Table B online). Notably, some of the Ta tumors surrounded by CIS were classified as T2, thus adding new information to clinical and pathologic classification.

Table 1 • Clinical data on disease courses and results of molecular classification

Sample	Previous tumors	Tumor analyzed	Subsequent tumors	Carcinoma *in situ*[a]	Reviewed histology[b]	Classifier[c]
Ta grade II tumors – no progression						
709-1		Ta gr2		no	Ta gr3	Ta
968-1		Ta gr2	1 Ta	no		Ta
934-1		Ta gr2		no		Ta
928-1		Ta gr2		no		Ta/T1
930-1		Ta gr2		no		Ta
Ta grade III tumors – no prior T1 tumor or CIS						
989-1		Ta gr3		no		Ta
1264-1		Ta gr3	3 Ta	no		Ta
876-5	4 Ta	Ta gr3		no		Ta
669-7	5 Ta	Ta gr3	4 Ta	no	Ta gr2	Ta
716-2	1 Ta	Ta gr3	2 Ta	no		Ta
Ta grade III tumors – no prior T1 tumor but CIS in selected site biopsies						
1070-1		Ta gr3	1 Ta	subsequent visit		Ta
956-2		Ta gr3	1 Ta	sampling visit		T2
1062-2		Ta gr3	1 T1	sampling visit		Ta
1166-1		Ta gr3		sampling visit		Ta
1330-1		Ta gr3		sampling visit		T2
Ta grade III tumors – a prior T1 tumor and CIS in selected site biopsies						
747-7	5 Ta, 1 T1	Ta gr3	3 Ta	sampling visit		Ta
112-10	7 Ta, 2 T1	Ta gr3	2 Ta, 4 T1	previous visit		Ta
320-7	1 Ta, 2 T1	Ta gr3	2 Ta	sampling visit		Ta
967-3	2 T1	Ta gr3	1 T1	sampling visit		Ta
T1 grade III tumors – no prior muscle-invasive tumor						
625-1		T1 gr3		no		T1
847-1		T1 gr3		no		T1
1257-1		T1 gr3		sampling visit		T1
919-1		T1 gr3		no		T1
880-1		T1 gr3	4 Ta	no		T1
812-1		T1 gr3		no		T1
1269-1		T1 gr3		no	no review	T1
1083-2	1 Ta	T1 gr3		no	no review	T1
1238-1		T1 gr3	1 Ta, 1 T2+	no		T1
1065-1		T1 gr3		subsequent visit	no review	T1
1134-1		T1 gr3	3 T1	sampling visit	T2 gr3	T1
T2+ grade III/IV tumors – only primary tumors						
1164-1		T2+ gr4		no	T2+ gr3	T1
1032-1		T2+ gr?		nd	no review	T2
1117-1		T2+ gr3		nd		T2
1178-1		T2+ gr3		nd		T2
1078-1		T2+ gr3		nd		T2
875-1		T2+ gr3		no		T2
1044-1		T2+ gr3	1 T2+	nd		T2
1133-1		T2+ gr3		nd		T2
1068-1		T2+ gr3		no		T2
937-1		T2+ gr3		nd	no review	T1

[a]Carcinoma *in situ* detected in selected site biopsies at the time of sampling tumor tissue for the arrays or at previous or subsequent visits. [b]Tumors were reviewed by a single uropathologist, and any difference from the routine classification is listed. [c]Molecular classification in the training set using 38 genes in cross-validation loops. nd, not determined.

We applied the classifier to an independent test set of 68 samples (see Web Table C online) analyzed on a different oligonucleotide array platform with other probes and different probe-set format. In light of the different array format, it was notable that the classifier 'correctly' classified (that is, included the histopathological classification) 84% of the Ta tumors, 50% of the T1 tumors and 74% of the T2+ tumors (see Web Table D online). Although some tumors from all stages were classified differently from conventional histopathological classification, this seems at least for Ta tumors to provide additional predictive information. Samples that the classifier grouped as T1 or T2 but that were classified histopathologically as Ta had a significantly ($P < 0.005$, Fisher's exact test) higher likelihood of disease progression or solid-tumor growth compared with those classified correctly (Table 3). This predictive property of the gene set may have considerable clinical

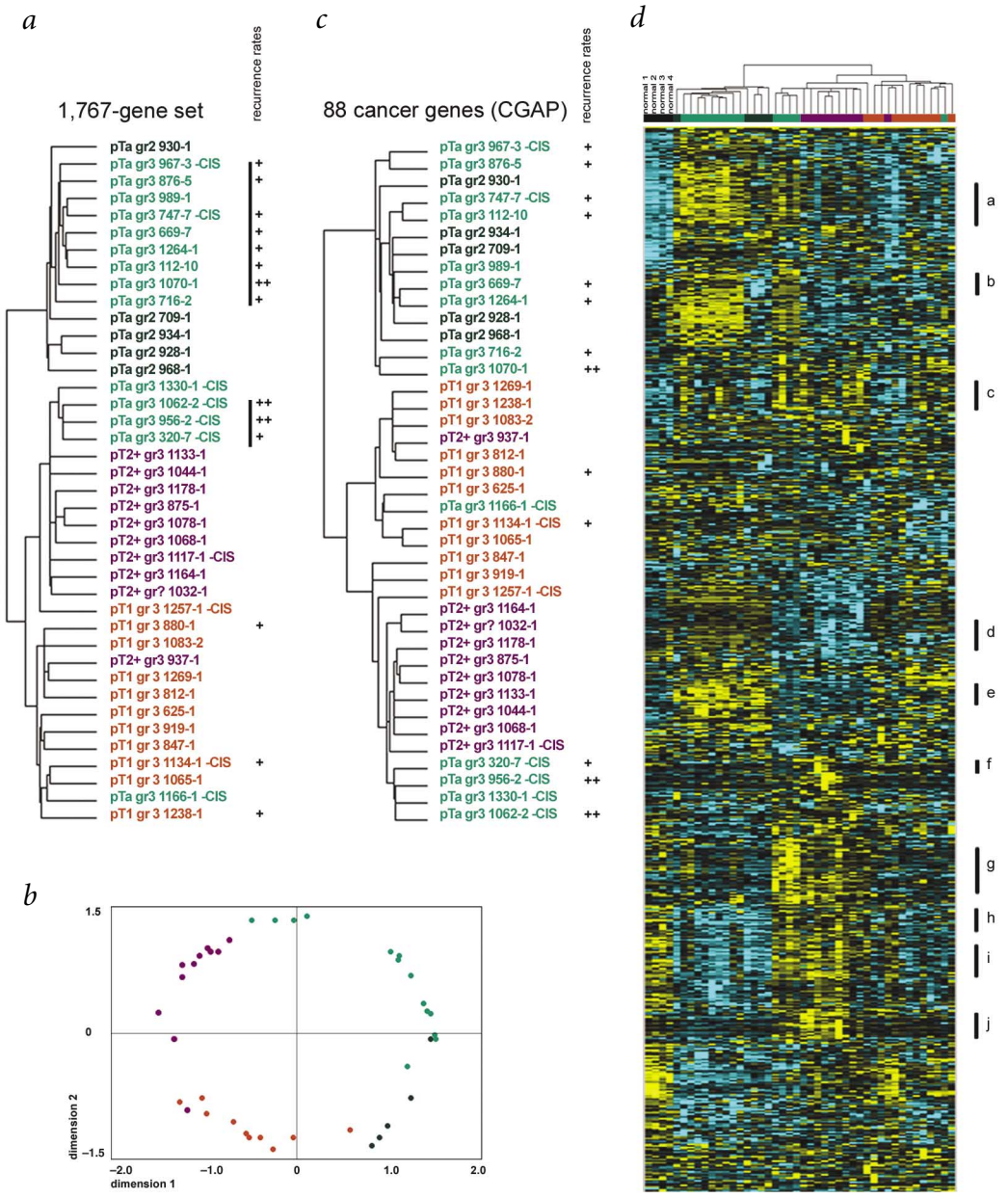

Fig. 1 Two-way hierarchical clustering and multidimensional scaling analysis of gene expression data from 40 bladder tumor biopsy samples. ***a***, Tumor cluster dendrogram based on the 1,767-gene set. The presence of CIS is noted after the sample names. Tumor recurrence rates are shown to the right of the dendrogram as + and ++, indicating medium and high recurrence rates, respectively; no sign indicates no recurrence or moderate recurrence. ***b***, Two-dimensional plot of multidimensional scaling analysis of the 40 tumors based on the 1,767-gene set. The color code identifies the tumor samples from the cluster dendrogram (Fig. 1*a*). ***c***, Tumor cluster dendrogram based on 88 cancer-related genes from CGAP. ***d***, Two-way cluster analysis diagram of the 1,767-gene set. Each row in the diagram represents a gene and each column a tumor sample. The color saturation represents differences in gene expression across the tumor samples; yellow indicates higher than the median expression (black), and blue indicates lower than median expression. The color intensity indicates degree of gene regulation. The sidebars to the right of the diagram represent gene clusters a–j. Normal 1–3 on the left side indicate the three normal biopsy samples, and normal 4 indicates the pool of biopsy samples from 37 individuals. There were two principal branches, one containing the superficial Ta tumors and the other containing the invasive T1 and T2+ tumors. In the superficial branch, two sub-clusters of tumors were identified, one containing eight tumors that had frequent recurrences and one containing three of the five Ta grade 2 tumors that had no recurrences. In the invasive branch, four Ta grade 3 tumors clustered tightly with the muscle-invasive T2+ tumors. These tumors showed concomitant CIS in the surrounding mucosa, indicating that this sub-fraction of Ta tumors had some of the more aggressive features of muscle-invasive tumors.

importance. Stage T1, which is an intermediate stage having both superficial and submucosa invasive components, seems difficult to classify using expression profiling.

We also tested whether the protein products of the classifying genes had similar classifying potential using immunohistochemistry, and compared our molecular classifier to the expression of the tumor stage–related proteins p53, Her2 and Ki-67. We immunostained a bladder tumor tissue array (n = 137) for Smad6 and cyclin G2 (Ta/T1 classifiers). Cyclin G2 was expressed at a lower level in T2 than in Ta tumors at both the RNA ($P < 0.0006$) and the protein levels ($P < 0.0009$). Fewer samples in the T2 group stained strongly for Smad6 than in the Ta group (see Web Fig. D online). For both cyclin G2 and Smad6, individual samples did not always show a correlation between RNA and protein level, as previously addressed[21]. The molecular tumor classifications correlated significantly to the expression of Ki-67 by immunohistochemistry ($P < 0.02$), but not significantly to p53 or Her2 staining (see Web Figs. E and F online).

We further tested an outcome predictor to determine if it was able to identify the likelihood of recurrence in individuals with superficial Ta tumors (see Web Table E online). We found that the optimal number of genes in cross-validation loops was 39 (75% of the samples were correctly classified, $P < 0.006$; see Web Fig. G and Web Table F online). From this group, we selected 26 genes (Fig. 3*b*) that were used in at least 75% of the cross-validation loops to constitute our final recurrence predictor. We tested the strength of the predictive genes by permutation analysis (see Web Table G online).

Our data on expression patterns that classify the benign and muscle-invasive bladder carcinomas can identify subgroups of bladder cancer such as Ta tumors with surrounding CIS, Ta tumors with a high probability of progression as well as recurrence and T2 tumors with squamous metaplasia. This could have implications for epithelial cancers in general, as these may be subdivided into a larger number of subclasses than has previously been expected. We found that the matrix-remodeling gene cluster was specifically expressed in tumors with the worst prognosis, namely the T2 tumors and tumors surrounded by CIS. For some

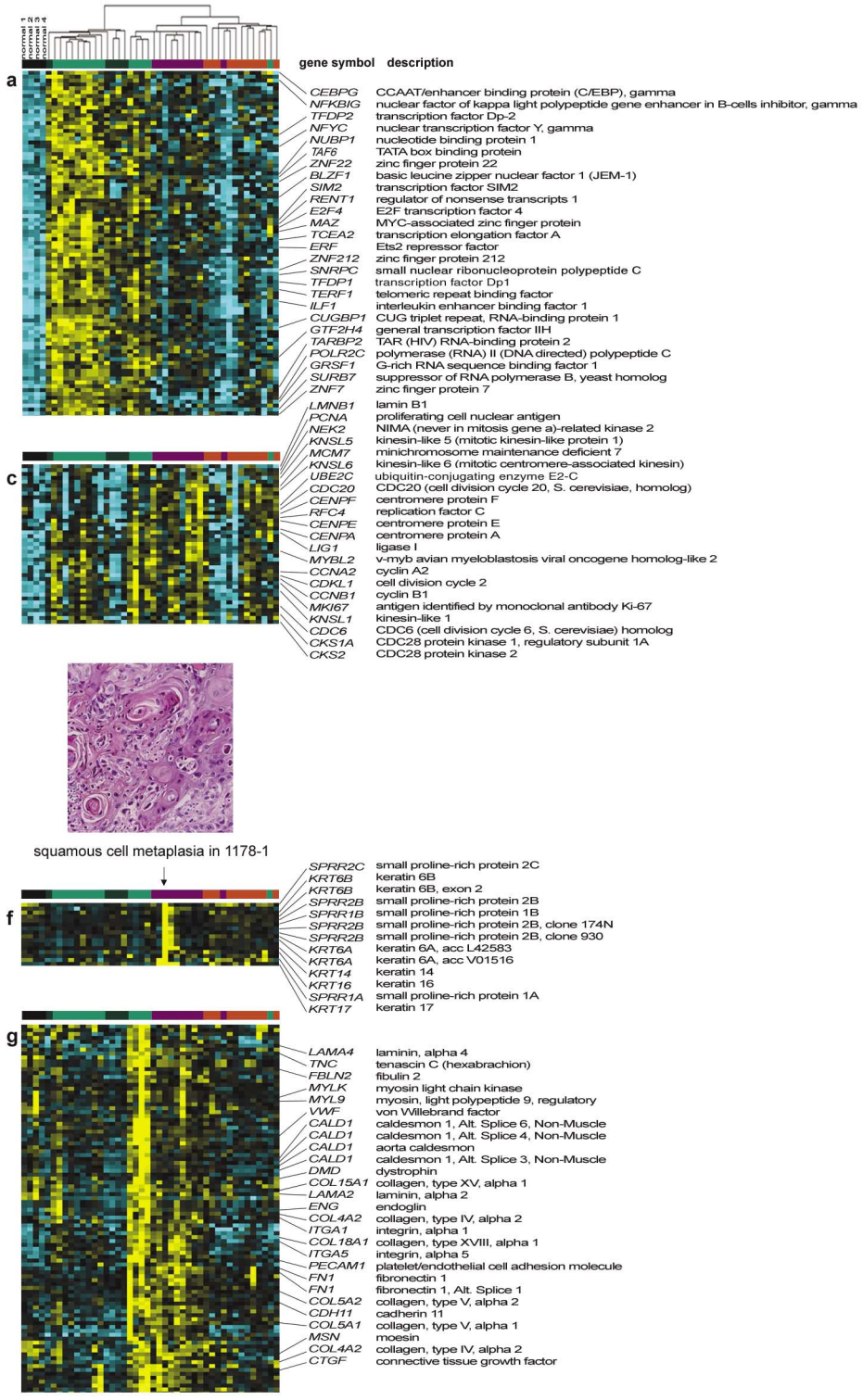

Fig. 2 Enlarged view of gene clusters A, C, F and G. The dendrogram at the top is identical to Fig. 1*a*. Cluster A, transcription factors and other nuclear genes; cluster C, genes involved in proliferation and cell-cycle control; cluster F, gene expression pattern and corresponding area with squamous metaplasia in urothelial carcinoma (yellow color indicates genes upregulated in samples 1178-1 and 875-1, the only two samples with squamous cell metaplasia); cluster G, genes involved in angiogenesis and matrix remodeling.

Table 2 • Summary of stage-related gene expression for functional gene clusters[a]

Tumor stage	Transcription	Nuclear processes	Proliferation	Matrix remodeling	Extracellular matrix	Immune system
Ta gr2	↑	nc	nc	nc	↓↓	↓
Ta gr3	↑↑↑	↑↑	↑↑	nc	↓↓	↓
T1 gr3	↑[b]	nc	↑↑[b]	nc	↓	↑[b]
T2 gr3	↑	nc	↑↑↑	↑↑↑	↑	↑
Ta gr3 + CIS	↑↑↑	↑↑	↑↑↑	↑↑↑	↑	↑

[a]For a detailed description of gene clusters, see Web Fig. A online. [b]An increase in gene expression was only found in about half of the samples analyzed. nc, no change.

of these genes, new small-molecule inhibitors already exist[22]. Introducing the presented classifiers for stage, progression and recurrence as well as the CIS-defining gene set into clinical routine requires a three-year prospective study, which we are currently carrying out based on custom microarrays holding the informative gene sets.

Methods

Biological material. We acquired 66 bladder tumor biopsy samples after the amount of tissue necessary for routine pathology examination had been removed. We froze the tumor samples immediately after surgery and stored them at –80 °C in a guanidinium thiocyanate solution. We graded each tumor according to previously published criteria[23], and a single pathologist then re-evaluated them. For normal urothelial reference samples, we used a pool of biopsy samples (from 37 individuals) as well as three single bladder biopsy samples from individuals with prostatic hyperplasia or urinary incontinence. Informed consent was obtained in all cases, and protocols were approved by the scientific ethical committee of Aarhus county.

RNA purification and cDNA preparation. We isolated total RNA from crude tumor biopsy samples using a Polytron homogenizer and the RNAzol B RNA isolation method (WAK-Chemie Medical GmbH). We used 10 µg total RNA as starting material for the cDNA preparation. We carried out the first- and second-strand cDNA synthesis using the SuperScript Choice System (Life Technologies) according to the manufacturer's instructions, except we used an oligo-dT primer containing a T7 RNA polymerase promoter site. We prepared labeled cRNA using the BioArray High Yield RNA Transcript Labelling Kit (Enzo). We used biotin-labeled CTP and UTP (Enzo) and unlabeled NTPs in the reaction. After the *in vitro* transcription reaction, we removed the unincorporated nucleotides using RNeasy columns (Qiagen).

Array hybridization and scanning. We fragmented 15 µg of cRNA at 94 °C for 35 min in a fragmentation buffer containing 40 mM Tris-acetate pH 8.1, 100 mM potassium acetate and 30 mM magnesium acetate. Before hybridization,we heated the fragmented cDNA in a 6×SSPE-T hybridization buffer (1 M NaCl, 10 mM Tris pH 7.6, 0.005% Triton) to 95 °C for 5 min and then to 45 °C for 5 min before loading onto the Affymetrix probe array cartridge (HuGeneFL). We then incubated the probe array for 16 h at 45 °C at constant rotation (60 r.p.m.). We carried out the washing and staining procedure in the Affymetrix Fluidics Station. We washed the probe array ten times in 6×SSPE-T at 25 °C and then four times in 0.5×SSPE-T at 50 °C. We stained the biotinylated cRNA with a streptavidin-phycoerythrin conjugate (final concentration 2 µg µl^{-1}; Molecular Probes) in 6×SSPE-T for 30 min at 25 °C and then washed it ten times in 6×SSPE-T at 25 °C. We scanned the probe arrays at 560 nm using a confocal laser-scanning microscope (Hewlett Packard GeneArray Scanner G2500A). We analyzed the readings from the quantitative scanning using the Affymetrix Gene Expression Analysis Software. We then amplified the signals using normal goat IgG as blocking reagent (final concentration 0.1 mg ml^{-1}; Sigma) and biotinylated goat antibody against streptavidin (final concentration 3 µg ml^{-1}; Vector Laboratories). We stained the samples with a streptavidin-phycoerythrin conjugate (final concentration 2 µg µl^{-1}; Molecular Probes) in 6×SSPE-T for 30 min at 25 °C and washed it 10 times in 6×SSPE-T at 25 °C. We then subjected the arrays to a second scan under similar conditions as described above.

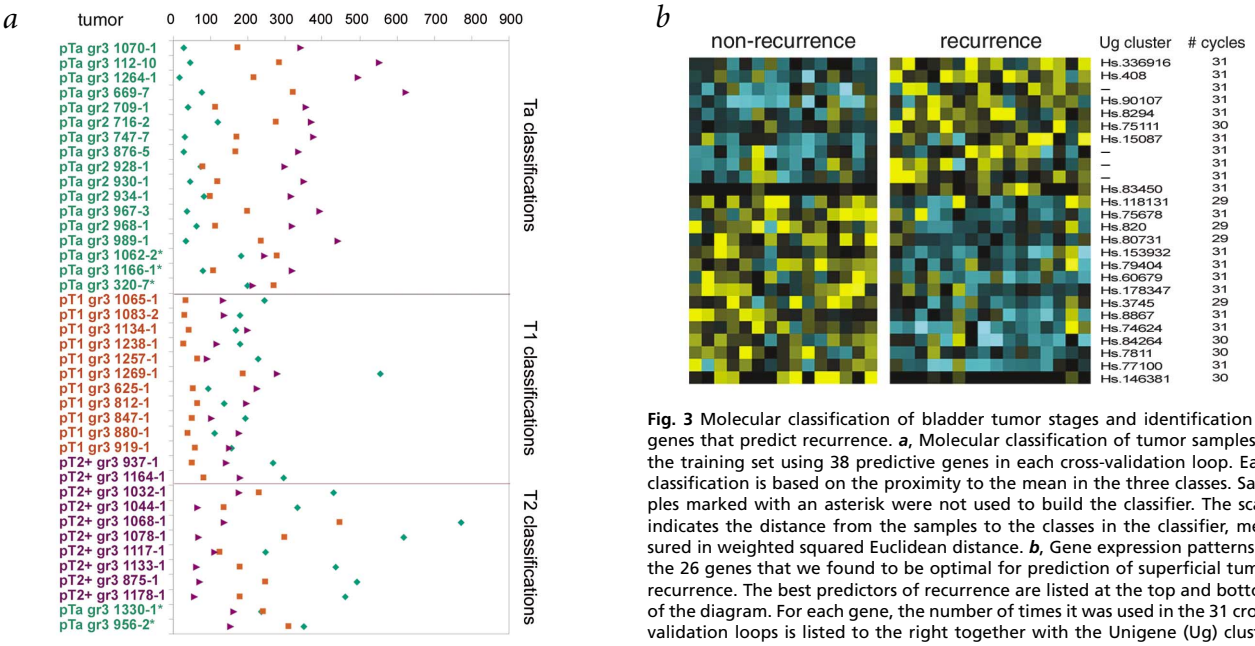

Fig. 3 Molecular classification of bladder tumor stages and identification of genes that predict recurrence. ***a***, Molecular classification of tumor samples in the training set using 38 predictive genes in each cross-validation loop. Each classification is based on the proximity to the mean in the three classes. Samples marked with an asterisk were not used to build the classifier. The scale indicates the distance from the samples to the classes in the classifier, measured in weighted squared Euclidean distance. ***b***, Gene expression patterns of the 26 genes that we found to be optimal for prediction of superficial tumor recurrence. The best predictors of recurrence are listed at the top and bottom of the diagram. For each gene, the number of times it was used in the 31 cross-validation loops is listed to the right together with the Unigene (Ug) cluster number (see Web Table G online).

Table 3 • Prediction of upstaging for stage Ta tumors using the molecular classifier

Sample	Histopathology	Molecular classifier[a]	Upstaging[b]
815-1	Ta gr2	T1	
861-1	Ta gr2	Ta	
898-1	Ta gr2	Ta	
368-4	Ta gr2	Ta	
669-1	Ta gr2	Ta	
747-3	Ta gr2	T1/Ta	
833-2	Ta gr3	Ta	
368-7	Ta gr3	Ta	
576-6	Ta gr2	Ta	
576-7	Ta gr2	Ta	
997-1	Ta gr2	Ta	
997-2	Ta gr2	Ta	
837-2	Ta gr2	Ta	
794-3	Ta gr2	Ta	
150-6	Ta gr3[c]	T2/T1	solid tumor
865-1	Ta gr2	Ta/T1	T1 tumor 5 mo later
1083-1	Ta gr2	T1	T1 tumor 2 wk later
825-3	Ta gr3	Ta	T1 tumor 5 mo later
941-4	Ta gr3[c]	T1/Ta	T2 tumor 6 mo later
112-2	Ta gr3	Ta/T1	T1 tumor 6 mo later
795-13	Ta gr2	Ta/T1	T1 tumor 4 mo later
686-3	Ta gr2	T1/Ta	T1 tumor 6 mo later
347-7	Ta gr3	Ta	T1 tumor 5 mo later
679-2	Ta gr2	T1/Ta	T2 tumor 30 mo later
1336-2	Ta gr1	Ta	T1 tumor 5 mo later

[a]Molecular classification of independent test samples using the 32-gene classifier. Two classifications are listed when the difference between the distances to the two closest groups is below 5%. [b]Individuals who developed a higher stage tumor later in the disease course. [c]Solid Ta tumors have an unfavorable disease course. All other Ta tumors in the table are papillomas.

Class discovery using hierarchical clustering. We scaled all microarray results to a global intensity of 150 units using the Affymetrix GeneChip software. Other ways of array normalization exist[24], but using the dCHIP approach did not change the expression profiles of the obtained classifier genes in this study (results not shown). For hierarchical cluster analysis and molecular classification procedures, we used expression-level ratios between tumors and the normal urothelium reference pool, which were calculated using the comparison analysis implemented in the Affymetrix GeneChip software. To avoid expression ratios based on saturated gene probes, we used the antibody-amplified expression data for genes with a mean Average Difference value across all samples below 1,000 and the non-amplified expression data for genes with mean Average Difference value across all samples equal to or above 1,000. We applied different filtering criteria to the expression data to avoid including in the data analysis those genes that did not vary or that were not highly expressed. First, we selected only genes whose expression was significantly different between the normal reference pool and at least three tumor samples. Second, we selected only genes with at least three 'Present' calls across all samples. Third, we eliminated genes with variation of less than 2 s.d. across all samples. The filtered gene set contained 1,767 genes. We carried out two-way hierarchical agglomerative cluster analysis using the Cluster software[25]. We used average linkage clustering with a modified Pearson correlation as similarity metric. Genes and arrays were median-centered and normalized to the magnitude of 1 before cluster analysis. We used the TreeView software for visualization of the cluster analysis results[25]. We carried out multidimensional scaling on median-centered and normalized data using an implementation in the SPSS statistical software package.

Tumor-stage classifier. We based the classifier on the log-transformed expression-level ratios. For these transformed values, we used a normal distribution with the mean dependent on the gene and the group (Ta, T1 and T2) and the variance dependent on the gene only. For each gene, we calculated the variation within the groups (W) and the three variations between two groups (B(Ta/T1), B(Ta/T2), B(T1/T2)) and used the three ratios B/W to select genes that had at least one high B/W ratio. To classify a sample, we calculated the sum over the genes of the squared distance from the sample value to the group mean, standardized by the variance. Thus, we calculated a distance to each of the three groups and classified the sample as belonging to the closest group. When calculating these distances, we estimated the group means and the variances from all the samples in the training set excluding the sample being classified.

Validation of the tumor-stage classifier. We validated the performance of the classifier using another set of bladder tumor expression data obtained from customized oligonucleotide Affymetrix GeneChips carrying PM probes only. First, we translated all accession numbers on both oligonucleotide microarrays into Unigene clusters and selected those gene probes present on both arrays (4,416 probe-sets). To make comparisons between the two microarray types, we used only the PM probe values from the original data set. We rescaled all the log (average PM) values and used the pool of normal bladder biopsy samples from 37 individuals, which were analyzed on both array platforms, to calculate log fold-change expression values. We recalculated the group means and the variances for each gene used in the classifier and based the classification on 29 genes from the optimal classifier in the cross-validation step for the original data set. We calculated the distances to each of the three groups for the new samples and classified them as belonging to the closest group.

Recurrence prediction using a supervised-learning method. We generated Average Difference values using the Affymetrix GeneChip software and set all values below 20 to 20 to avoid very low and negative numbers. We only included genes that had a 'Present' call in at least seven samples and genes that showed intensity variation of max–min > 100 and max/min > 2. The values were log-transformed and rescaled. We used a supervised-learning method essentially as described[11]. We selected genes using *t*-test statistics and cross-validation and classified samples as described above.

Immunohistochemistry. We prepared tumor tissue microarrays essentially as described[26], with four representative 0.6-mm paraffin cores from each study case. We carried out immunohistochemical staining using standard highly sensitive techniques after appropriate heat-induced antigen retrieval. Primary polyclonal goat antibodies against Smad6 (S-20) and cyclin G2 (N-19) were from Santa Cruz Biotechnology. Antibodies to p53 (monoclonal DO-7) and Her-2 (polyclonal anti-c-erbB-2) were from Dako A/S. Ki-67 monoclonal antibody (MIBI) was from Novocastra Laboratories. An experienced pathologist who was unaware of array results scored the staining intensity at four levels (negative, weak, moderate and strong), considering both color intensity and number of stained cells.

GEO accession numbers. Array data was deposited at the Gene Expression Omnibus (National Center for Biotechnology Information) with accession numbers GSM2474 through GSM2544. The array data is also found in series with accession number GSE89 (tumor stage classification) and GSE88 (recurrence prediction).

Note: Supplementary information is available on the Nature Genetics website.

Acknowledgments

We thank K.Y. Jonsdottir for computational assistance, H. Steen, B. Pytlick, J.C. Djurhuus, B. Stougaard and B. Devantié for excellent assistance, and the staff at the Departments of Urology, Clinical Biochemistry and Pathology at Aarhus University Hospital. We acknowledge support from the Karen Elise Jensen Foundation, The Danish Cancer Society, EOS Biotechnology, the John and Birthe Meyers Foundation, The Institute of Experimental Clinical Research, The University and County of Aarhus and the Danish Research Council.

Competing interests statement

The authors declare competing financial interests. Details accompany the paper on the Nature Genetics website (http://www.nature.com/naturegenetics).

Received 9 October; accepted 6 November 2002.

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