Identifying Superficial, Muscle-Invasive, and Metastasizing Transitional Cell Carcinoma of the Bladder: Use of cDNA Array Analysis of Gene Expression Profiles

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ABSTRACT

Purpose: Expression profiling by DNA microarray technology permits the identification of genes underlying clinical heterogeneity of bladder cancer and which might contribute to disease progression, thereby improving assessment of treatment and prediction of patient outcome.

Experimental Design: Invasive (20) and superficial (22) human bladder tumors from 34 patients with known outcome regarding disease recurrence and progression were analyzed by filter-based cDNA arrays (Atlas Human Cancer 1.2; BD Biosciences Clontech) containing 1185 genes. For 9 genes, array data were confirmed using real-time reverse transcription-PCR. Additionally, Atlas array data were validated using Affymetrix GeneChip oligonucleotide arrays with 22,283 human gene fragments and expressed sequence tags sequences in a subset of three superficial and six invasive bladder tumors.

Results: A two-way clustering algorithm using different subsets of gene expression data, including a subset of 41 genes validated by the oligonucleotide array (Affymetrix), classified tumor samples according to clinical outcome as superficial, invasive, or metastasizing. Furthermore, (a) a clonal origin of superficial tumors, (b) highly similar gene expression patterns in different areas of invasive tumors, and (c) an invasive-like pattern was observed in bladder mucosas derived from patients with locally advanced disease. Several gene clusters that characterized invasive or superficial tumors were identified. In superficial bladder tumors, increased mRNA levels of genes encoding transcription factors, molecules involved in protein synthesis and metabolism, and some proteins involved into cell cycle progression and differentiation were observed, whereas transcripts for immune, extracellular matrix, adhesion, peritumoral stroma and muscle tissue components, proliferation, and cell cycle controllers were up-regulated in invasive tumors.

Conclusions: Gene expression profiling of human bladder cancers provides insight into the biology of bladder cancer progression and identifies patients with distinct clinical phenotypes.

INTRODUCTION

Bladder cancer is the fifth most common solid tumor in man, with an overall estimated annual incidence of 57,400 new cases in the United States (1). Of these, ~70% are superficial at the time of initial presentation, whereas 30% of patients are diagnosed with muscle-invasive disease. Despite treatment, about half of patients with muscle-invasive bladder cancer will develop distant cancer recurrence and ultimately succumb to their disease, leading to 12,100 deaths annually (2).

Superficial bladder tumors, which are removed by transurethral resection, are characterized by frequent tumor recurrences (3). Molecular analysis of multiple synchronous tumors and recurrences reveal that the vast majority of these are clonally related (4–8). Moreover, some muscle-invasive bladder carcinomas can arise from independently transformed progenitor cells (9). A small group of superficial bladder tumors (5–30%, depending on stage and grade) will progress to muscle invasive disease (10–14). Curative treatment of invasive tumors requires radical cystectomy with urinary diversion. Depending on the tumor stage at cystectomy 30–70% of patients will develop distant cancer recurrence. Some patients with locally advanced disease or lymph node metastasis may benefit from adjuvant chemotherapy to treat (micro-) metastatic disease (15–17). Thus, two major events determine the outcome of patients with bladder cancer: (a) progression from superficial to invasive disease, and (b) the development of metastases.

The current Tumor-Node-Metastasis classification system of solid tumors is based on the anatomical spread of the disease, which to some degree reflects the biological aggressiveness of cancer. However, clinical experience shows a broad range of behavior among tumors at the same stage. Recent studies have focused on cancer-associated molecular alterations because the genotype might better indicate the clinical phenotype of a tumor rather than a sample taken at a single time point. More detailed molecular insights into cancer biology are expected to eventually provide a classification allowing optimized therapy of individual patients.

For this purpose, several individual molecular markers, most of which are involved in cell cycle regulation, have been studied previously. Indeed, immunohistochemical detection of the tumor suppressor genes p53 and Rb, as well as cell cycle
regulator p21\textsuperscript{Waf1/CIP1}, appeared to correlate moderately well with bladder cancer recurrence after radical cystectomy in retrospective studies (18–21). However, multiple genetic alterations are required for transformation of a normal cell into the malignant and finally metastatic phenotype. Therefore, assessment of multiple markers may better describe the biological phenotype of a particular cancer.

Several previous studies have applied microarray technology to gain insight into the changes in expression profiles occurring during different stages of bladder cancer disease. Cell lines and clinical tissue samples have been used for monitoring of expression of thousands of genes. These investigations were focused on the role of histopathological stage, tumor grade, and proliferation activity in bladder cancer cell lines and tissues (22–26).

In this study, the expression of 1176 cancer-related genes was studied using a filter cDNA array to identify genes and gene clusters involved in bladder cancer progression from superficial to invasive and metastatic disease. Several clusters of functionally related genes where identified that were differentially expressed between invasive and superficial bladder tumors. Moreover, invasive tumors could be subdivided into two groups with different outcomes based on their gene expression patterns. Additional elucidation of such genetic differences might improve the prediction of the clinical course of disease allowing optimized treatment the bladder cancer patients.

### MATERIALS AND METHODS

#### Patients and Tumor Specimens

Overall, 42 transitional cell carcinoma specimens derived from 34 patients who underwent surgery at the Department of Urology, Heinrich-Heine University (Düsseldorf, Germany) were examined. Fourteen patients had superficial (T\textsubscript{a}, T\textsubscript{1}) and 20 had muscle-invasive (≥pT\textsubscript{2}) disease. The tumors were staged according to the current Tumor-Node-Metastasis classification system by Union International Contre Cancer and graded according to Bergkvist et al. (27). Patient and tumor characteristics, as well as clinical outcome, are summarized in Tables 1 and 2.

The 22 superficial papillary transitional cell carcinomas from 14 patients included pTaG1 (5), pTaG2 (14), pT1G2 (2), and pT1G3 (1) tumors. One superficial tumor was obtained during nephroureterectomy and all others by transurethral resection between October 1999 and September 2000. Each papillary-shaped tumor was cut at the base to avoid coagulation of the specimen. To compare multifocal and recurrent bladder carcinomas of 7 patients, 2 and in one case 3, tumors were examined. These included (a) two primary tumors and the first recurrence, (b) five multifocal tumors (including one multifocal recurrence), and (c) one synchronous ureter and bladder tumor. No patient with superficial bladder cancer has progressed to muscle-invasive disease after a mean follow-up period of 32 months.

Specimens of locally advanced bladder cancer were obtained from 20 patients during radical cystectomy between August 1993 and December 2000. Of four muscle-invasive tumors (P1, P35, P36, P37) specimens from different areas, e.g., superficial or deeper layer of the bladder wall, were examined. These tumors were macro-dissected into two to four counterparts containing different proportions of tumor to stroma cells. In three of these and one other case (P24), corresponding normal mucosa of the bladder was available. Half of the 20 patients recurred after a mean time period of 9 months and subsequently died of disease. Eight patients remain alive after a mean follow-up of 75 months. Two patients died perioperatively.

#### Tissue Sample Preparation

Immediately after surgical resection, tumors were macro-dissected. Each bladder cancer sample was confirmed as representative by adjacent fresh frozen and paraffin-embedded sections of cystectomy and transurethral resection specimens, respectively, then frozen in liquid nitrogen and stored at −80°C until RNA extraction. H&E-stained sections from tumor specimens and normal mucosa samples were examined to assess the relative proportion of tumor cells, benign epithelium, stroma, and lymphocytes.

#### Bladder Cancer Cell Lines

The origin and culture of transitional cell carcinoma cell lines T24, VM Cub 1, and VM Cub 3 have been described previously (28). Cells were harvested at 70% confluence for RNA extraction.

#### Total RNA Isolation and cDNA Probe Synthesis

Total RNA from tissue specimens and cell lines was extracted by the guanidine-isothiocyanate method, followed by phenol/chloroform extraction and DNase treatment using the recommended protocol for the Atlas Pure Total RNA Labeling System (BD Biosciences Clontech, Heidelberg, Germany). Total RNA was quantified using UV spectrophotometry (Photometer ECOM 6122; Eppendorf AG, Hamburg, Germany), and the quality and integrity was confirmed by electrophoresis of 0.5 μg of isolated RNA in 1% formamide agarose gels. Additionally, RNA specimens were analyzed by microcapillary electrophoresis on LabChips using the Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Boeblingen, Germany) following the manufacturer’s instructions.

For first-strand cDNA synthesis, 5–10 μg of native total RNA were mixed with 1 μl of coding sequence primer mix (0.02 μl; BD Biosciences Clontech), incubated at 70°C for 4 min followed by 4 min at 50°C. Master mix containing 2 μl of 5× reaction buffer, 1 μl of 10× deoxynucleoside triphosphate mix (5 mm each of dCTP, dGTP, dTTP, and dATP), 0.5 μl of 100 mm DDT, 3 μl of [α-32P]dATP (10 μCi/μl; 3000 Ci/mmol; Amersham Biosciences Europe GmbH, Freiburg, Germany), and 1 μl of Moloney murine leukemia virus reverse transcriptase (100 units/μl; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was added to each template. After incubation at 50°C for 35 min, the reaction was terminated using a mixture of glycogen and EDTA [10× Termination mix: 0.1 μl EDTA (pH 8.0), 1 mg/ml glycogen; BD Biosciences Clontech], the labeled cDNA probes were purified and probe incorporation assessed by scintillation counting.

#### Hybridization and Image Analysis

Atlas Human Cancer 1.2 Array nylon membranes (BD Biosciences Clontech) containing 1185 cDNA spots corresponding to 1176 known human genes and 9 human housekeeping genes, as well as genomic DNA spots and positive and negative controls, were probed with labeled cDNAs by overnight hybridization at 68°C using ExpressHyb Hybridization Solution (BD Biosciences Clontech). Briefly, radiolabeled probes were heat denatured and then added to 5-ml aliquots of hybridization buffer containing 100 μg/ml heat-denatured sheared salmon.
testes DNA (Life Technologies, Inc., Karlsruhe, Germany). The final probe concentration was $1 \times 10^7 \text{ cpmp/ hybridization.}$ For all samples, expression profiles were averaged from two independent hybridization experiments performed with separate array filters. Identical batches of gene-specific primer mix and membranes were used to exclude possible variations in cDNA probe synthesis and hybridization. After extensive washing (three times with 2× SSC, 1% SDS, and once with 0.1× SSC, 0.5% SDS each for 30 min at 68°C, once with 2× SSC for 10 min at room temperature), the membranes were exposed to PhosphoImager screens (BAS-1500; Fuji, Raytest, Germany).

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* Site of tumor recurrence: Br, brain; L, liver; Ln, lymph nodes; B, bone; Lu, lungs; Pl, pleura; Pe, peritoneum; n/a, not available; D, perioperative death; LN, lymph node metastasis; MU, mucosa.

* A, alive; DOD, dead of disease.

| Table 2 | Clinicopathological information: superficial tumors* |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Case    | Age (yrs)       | Gender | Type | Stage | Grade | Time to recurrence (months) | Recurrence | Follow-up (months) | Note               |
| 10      | 68              | M      | Primary | Tsa | G2     | 5               | TaG2          | 39              | Multifocal       |
| 11      | 75              | M      | Primary | Tsa | G2     | 5               | TaG2          | 39              | Multifocal       |
| 12      | 48              | M      | Primary | Tsa | G2     | 5               | TaG2          | 39              | Multifocal       |
| 13_2    | 61              | M      | Recurrence | Tsa | G2     | 13              | TaG2          | 39              | Multifocal       |
| 14_1    | 64              | M      | Primary | Tsa | G2     | 5               | TaG2          | 39              | Multifocal       |
| 14_2    | 64              | M      | Primary | Tsa | G2     | 5               | TaG2          | 39              | Multifocal       |
| 15_1    | 75              | M      | Primary | Tsa | G1     | 15              | TaG2          | 33              | Multifocal       |
| 15_2    | 75              | M      | Primary | Tsa | G1     | 15              | TaG2          | 33              | Multifocal       |
| 16_1    | 73              | M      | Primary | Tsa | G2     | 5               | TaG2          | 39              | Multifocal       |
| 17_1    | 87              | M      | Recurrence | Tsa | G2     | No              | TaG1          | 31              | Multifocal       |
| 17_2    | 87              | M      | Recurrence | Tsa | G2     | No              | TaG1          | 31              | Multifocal       |
| 18      | 73              | M      | Primary | Tsa | G1     | 31              | TaG1          | 31              | Tumor recurrence of case no. 19 |
| 19      | 65              | F      | Primary | Tsa | G2     | 4               | TaG2          | 37              | Tumor recurrence of case no. 19 |
| 20      | 66              | M      | Primary | Tsa | G2     | 22              | TaG2          | 37              | Tumor recurrence of case no. 19 |
| 21      | 71              | M      | Recurrence | Tsa | G1     | No              | TaG2          | 37              | Tumor recurrence of case no. 19 |
| 22      | 68              | M      | Primary | Tsa | G3     | 7               | TaG2          | 34              | Tumor recurrence of case no. 19 |
| 40_1    | 69              | M      | Recurrence | Tsa | G2     | 7               | TaG2          | 34              | Tumor recurrence of case no. 19 |
| 40_2    | 69              | M      | Recurrence | Tsa | G2     | 7               | TaG2          | 34              | Tumor recurrence of case no. 19 |
| 38      | 77              | M      | Recurrence | Tsa | G1     | 8               | TaG2          | 26              | Tumor recurrence of case no. 19 |

* There was no patient with tumor progression to muscle-invasive disease.
Atlas Array Data Analysis. Radioactive intensity of each spot on the membrane was linearly digitized to gray levels with a pixel size of 100 μm (PhosphoImager) and recorded using a commercially available image analysis software (TINA; Fuji). Data were obtained as a list of intensity values for all measured positions on the array. All values derived from image analysis were corrected for background using Excel 97 software (Microsoft). Normalization of signal intensities obtained from different hybridization experiments was based on the sum of background-subtracted signal data of all expressed genes and was performed to account for differences between labeling and quality of RNA samples. Such total intensity normalization method relies on the assumption that quantities of initial mRNA are the same for all samples. Under this assumption, a normalization factor can be calculated and used to re-scale the intensity for each gene in the array (29). Absolute values corresponding to abundance of RNA in the probe were normalized according to mean value (after normalization the mean value of signal intensities in each array was equal to 1.0). The resulting data table and raw data are available from our web site (see data files 1, 2).4

Reproducibility of experiments was verified by comparing one hybridization with the same probe on two independent arrays. Identical batches of gene-specific primer mix and membranes were used to exclude possible variations in cDNA probe synthesis and hybridization. The results showed good reproducibility with correlation coefficients from 0.9 to 0.98. Moreover, the notch group protein gene represented by two different clones (A02g and A05 h) displayed similar expression profiles for the two clones in all samples.

Hierarchical Clustering. Mean values were calculated for each gene from the invasive and superficial groups of samples. Weak signals on the membrane close to background result in an unfavorable signal-to-noise ratio and show low reproducibility between experiments. Ratios in gene expression between different samples based on such values may be very high even when there is no significant difference in the expression level of the corresponding genes (29). Therefore, only genes with a mean expression value of >0.7, either in the invasive or in the superficial group, were used for additional analysis (n = 270). The normalized values of 270 genes were log transformed, and data for each gene were mean-centered and normalized. Data for each sample were also mean-centered. The hierarchical clustering program (version 3.1) developed by Eisen et al. (30) was applied to group samples on the basis of overall similarities in their gene expression patterns, as well as to group genes on the basis of similarity of their expression levels in all samples. Results were displayed with the TreeView program, version 1.6.6 (Ref. 30; Fig. 1). Red color was used to show genes with expression levels greater than the mean and green for expression levels lower than the mean.

Oligonucleotide Array Hybridization and Analysis. Aliquots of total RNA of 9 tumors were subjected to additional analysis using the Affymetrix HG-U133A array. Three of these samples were from superficial tumors (p10, 11, and 12), 3 from invasive tumors of patients with long-term disease-free survival (p1, 30, and 34), and 3 from invasive tumors of patients with rapid tumor progression and cancer-related death (p23, 27, and 36). The HG-133A array contains 22,283 human gene fragments and expressed sequence tag sequences.

Total RNA (5 μg) was used in Affymetrix oligonucleotide array hybridization. The whole experimental procedure was performed according to standard Affymetrix protocols, and hybridizations were done in the core facility at the Department of Chemical Oncology, Heinrich-Heine University. Synthesized and fragmented cRNAs were analyzed by electrophoresis using LabChips and the Agilent 2100 bioanalyzer.

Arrays were scanned at 560 nm using a laser-scanning microscope (Agilent GeneArray Scanner G2500A; Agilent Technologies GmbH). To normalize for sample loading and staining variation, the average of the fluorescent intensities of all probe sets on an array were scaled to a constant target signal intensity (target signal factor 100) for all arrays used. Scaling factor between arrays was <2.

Expression profile data were collected and analyzed using Affymetrix Microarray Suite 5.0. To identify differentially expressed genes between superficial and invasive tumors, the ratio of average expression level for each gene between the two groups (invasive to superficial) was calculated. For this analysis, raw data as scaled by the Affymetrix software were used (see data file 4 from our web site).4 Low values corresponding to signal intensities close to background and defined as ‘A’ (absent) were not changed. When the intensity of the detected genes is close to background, it is not accurate to calculate the ratio. Therefore, ratios for genes that are not expressed in one sample group but expressed in the second one should be considered as relative. Because the empirical minimum for detection of a signal on the Affymetrix array by use of target signal factor = 100 is 30, the cutoff value for the average gene expression levels was set to 100.

To compare Atlas array hybridization data obtained for 34 patients with 22 superficial and 20 invasive bladder cancers, data obtained from Affymetrix platform for the subset of 9 bladder cancers was filtered to include only genes in common with both arrays. For filtering, GenBank accession numbers have been used. Of 1185 genes listed on the Clontech array, 1164 corresponding genes could be identified on the Affymetrix HG-U133A array. There are 8 genes on the Human Cancer 1.2 array, which are present twice, and one gene with three positions. This data file is available from our web site (see data files 4, 5).4

Statistical Analysis. Standard statistical procedures, including the Wilk-Shapiro and the unpaired Student’s t test, were used. Correlation in gene expression between data obtained from Affymetrix and Clontech platforms were analyzed by Spearman’s rank test. All reported tests were two-sided, and P < 0.05 was considered significant.

Beisbarth et al. (31) systematically analyzed array data from radioactive hybridization array experiments with regard to comparability of different experiments and reproducibility. The authors concluded that a 2-fold change can be considered significant given a high reproducibility with correlation coefficients of 0.9 as we did observe (see above). According to this and other studies (32–37), a change of at least factor 2 was considered significant.

Fig. 1  A two-way clustering analysis of bladder invasive and superficial bladder cancer tissues and cancer cell lines. Thirty-four samples and three cell lines are lined up on the horizontal axis, and 270 genes prefiltered from 1185 genes listed on Clontech Atlas human cancer 1.2 filter array are on the vertical axis (see “Materials and Methods”). Superficial and invasive tumors, as well as cell lines, were distinguished. Each cell in the color matrix represents the relative expression level of a single gene transcript in a single sample. Expression level was normalized per gene, and the relative value to the mean among 37 arrays is shown by color: red; transcript levels above the mean for the given gene across the samples, green; transcript levels below the mean for the given gene across the samples. The magnitude of deviation from the mean value is represented by color saturation. A, matrix representation of expression levels and the dendrogram of samples. B, expanded view of several gene clusters of interest named from top to bottom: a, integrin cluster; b–d, clusters of genes involved in nucleotide and nuclear acid metabolism, protein metabolism and synthesis, repair processes, cell growth and differentiation, and cell cycle control; e, stromal cluster; f, extracellular matrix remodeling proteins; g, cluster contains genes controlling cell cycle, and cell proliferation and growth.
Quantitative Real-Time Reverse Transcriptase-PCR (rtRT-PCR). rtRT-PCR was performed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA (3 μg) from 24 bladder tumor samples (superficial: 10, 12, 13I, 13II, 14I, 15I, 17I, 29, 38, 40I, 40II; invasive: 1, 2, 23, 24, 25, 26, 34, 35I, 36I, 36II, 37I, 37II) were reverse transcribed using random hexamer primer and Superscript II (Life Technologies, Inc.) as recommended by the manufacturer. Each cDNA mixture was diluted 1:8 in distilled H2O for subsequent PCR amplification using Assays-on-Demand Gene expression products (Applied Biosystems). Expression of the gene for cyclophilin A served as an internal control. Data were extracted and amplification plots generated with the ABI Sequence Detection System software. All amplifications were done in duplicates and threshold cycle (Ct) scores were averaged for subsequent calculations of relative expression values. Quantification of rtRT-PCR data was performed as follows. The Ct scores for genes of interest for each sample were normalized against Ct scores for the corresponding endogenous control gene (cyclophilin A; PPIA): Δ Ct = C t Target gene − C t PPIA. Values of Δ Ct detected by rtRT-PCR were compared with ln of expression obtained from array experiments (Clontech and/or Affymetrix) by calculating of Pearson correlation coefficient (r) and its corresponding P.

RESULTS
Gene Expression Profiling of Bladder Cancer. RNA isolated from 42 different human bladder tumors from 34 patients and from three bladder cancer cell lines were used in hybridization experiments with cDNA arrays containing 1176 cancer-associated and 9 housekeeping genes. Of these genes, 270 were expressed at significant levels either in the invasive or in the superficial tumor group and were subjected to hierarchical cluster analysis.

The unsupervised clustering algorithm groups both genes and tumors according to similarities in expression levels. Overall, samples divided into two major clusters which separated all 42 bladder cancers according to histopathological features (Fig. 1). Thus, invasive tumors were placed separately from the superficial tumors branch of the dendrogram. Expression profiles of three cancer cell lines examined were grouped together on a subbranch of the sample dendrogram within the superficial tumors branch. This branch also included a high-grade superficial invasive tumor (pT1G3) and a locally advanced tumor recurrence after an initial pT1G3 disease. A similar separation of tumor groups was also obtained using expression data from the full gene expression dataset (1185 genes; Fig. 2A).

Notably, unsupervised clustering also divided the muscle-invasive tumors into two major subgroups with disproportional representation of adverse clinicopathological features. Subgroup II (Fig. 2A, P8-P23, right) mainly contained patients with locally advanced disease or lymphatic spread and those who rapidly recurred after radical cystectomy. In contrast, 5 of 9 patients (56%; Fig. 2A, P39-P24, left) in subgroup I experienced long-term recurrence-free survival (mean, 66 months). Considering only those patients with organ-confined disease (pT2bNOG3), 1 of 5 compared with 4 of 4 tumors in the two different branches progressed.

We also compared gene expression profiles of different samples of multifocal superficial tumors and samples from different parts of invasive tumors (Fig. 2B). The same set of 270 genes, described above, was used in the hierarchical clustering algorithm. Although all 5 multifocal superficial bladder tumors were each clustered together, the synchronous ureter and bladder cancer (p14I & p14II, both pTaG2) were separated on the
dendrogram. Expression profiles of differently localized tumor specimens from invasive tumors (P1, P35, P36, and P37) were more similar to themselves than to samples from different tumors. The only exception was a part of tumor P36 containing a smaller proportion of tumor cells (data not shown). Expression profiles of samples corresponding to normal mucosa of patients with locally advanced tumors were clustered together but separately from their corresponding tumor specimens as a sub-branch in one invasive tumors branch.

There are 124 genes in the Clontech dataset with fold change of 2, 63 down, and 61 up. By use of an additional criterion for \( P \) of 0.05 (Student’s \( t \) test), 106 genes showed differential expression in invasive versus superficial tumors. Therefore, the false discovery rate (32) of the 106 probe set is 5.8%. For 48 genes, mRNA levels were increased in invasive versus superficial tumors, and 58 genes were decreased in invasive versus superficial tumors (see Supplementary Materials; Table 1). The clustering algorithm identified several clusters of genes that were differentially expressed between invasive and superficial tumors. These clusters included genes involved in nucleotide and nucleic acid metabolism, protein metabolism and synthesis, DNA repair, cell growth and differentiation, and cell cycle control. Clusters b, c, and d (Fig. 1) were expressed at higher levels in superficial tumors. In contrast, invasive tumors demonstrated higher expression of genes (clusters e and f; Fig. 1) encoding stroma proteins, proteins involved in extracellular matrix remodeling, as well as immune system proteins.

Clusters a and g consisted of genes that were expressed significantly higher in 6 invasive tumors compared with other invasive samples. These specimens, derived mainly from high-risk patients, were clustered together on a distinct branch of the sample dendrogram (Fig. 1). Cluster a contained genes related to cell cycle regulation, transcription, cell-matrix, and cell-cell adhesion. Cluster g contained genes controlling cell cycle, and cell proliferation and growth.

The gene expression profile of epithelial bladder tumor cell lines grown in vitro and the human bladder tumor samples did not show major differences. There were, however, several genes that were preferentially expressed in cell lines but not in tumor samples (see Supplement Cell Lines; web site as above). These genes were not included in the 270 gene set further used for classifying of tumors. The genes up-regulated in the examined cell lines were mainly involved with proliferation processes and cell growth.

**Affymetrix GeneChip Hybridization.** To further explore results from Atlas arrays, expression data obtained by Affymetrix GeneChip HG-U133A, containing 22,283 human genes and expressed sequence tags, were compared between 3 randomly selected superficial and 6 invasive tumors. Of 45–50% elements expressed, a total of 215 genes was differentially expressed in all 19 pairwise comparisons, as defined by criteria in the Affymetrix software. The expression levels of 105 mRNAs were increased in invasive tumors and of 110 mRNAs were increased in superficial tumors. Applying a more stringent 10-fold change as the cutoff, we generated a subset of 77 differentially expressed genes (list provided in Supplemental Table 2).

**Comparison of Gene Expression Data for Clontech and Affymetrix Platforms.** On the basis of the 270 genes identified by analysis of the Atlas data, a group of 117 genes whose...
corresponding measurements using the Affymetrix GeneChip significantly correlated in a Spearman’s rank test ($P < 0.05$) were identified (for detailed list see our web site “Affymetrix versus Clontech Data”). For the majority of 153 genes, however, the direct comparison of Atlas and the Affymetrix datasets revealed a lack of agreement between the two platforms. To illustrate the discrepancy between both platforms, scatterplots were used (Fig. 3). As illustrated in Fig. 3, some genes that appear up-regulated on the Clontech platform did not change or even show down-regulation on the Affymetrix platform and vice versa. A detailed comparison of Clontech and Affymetrix data are subject of a separate article.

Of the 117 corresponding genes, 41 were also differentially expressed between superficial and invasive tumors based on data from both platforms. We again applied the hierarchical clustering algorithm to group tumor samples and these 41 genes on the basis of their overall similarities (Fig. 4). Gene clusters, as well as sample groups, were similar to those obtained for the 270 genes from the Atlas arrays and clearly separated superficial and invasive tumors (Fig. 1). Moreover, the majority of muscle-invasive tumors of subgroup II (Fig. 2 A), characterized by adverse clinicopathological features, remained as a separate branch.

Validation Using rtRT-PCR. To confirm the array data, 24 of the initial 42 bladder cancers were randomly chosen, and rtRT-PCR was performed for the 11 genes listed in Table 3. With the exception of only 2 of the 11 genes, there was a significant correlation between the Clontech and/or Affymetrix expression data. Some gene transcripts were represented by more than one probe set on Affymetrix arrays and each detected similar relative levels of expression, e.g., different variants of the decorin gene (Table 3). However, the extent of change detected by the different methods varied.

Two of the genes validated, decorin and stratifin, are...
differentially expressed between the two major subgroups of muscle-invasive bladder cancer. Fig. 5 illustrates the expression of decorin and stratifin as determined by either rtRT-PCR or Atlas array analysis. Despite the small sample size, the difference in expression between the subgroups was highly significant for stratifin and approached statistical significance for decorin.

The rtRT-PCR expression data of two genes, p21 Waf1/Cip1 and SOCS3, did neither correlate with Clontech nor with Affymetrix expression data for the selected samples. Despite three different probe sets, the Affymetrix GeneChip failed to detect expression of signal transducers and activators of transcription-activated signal transducers and activators of transcription inhibitor 3 (SOCS3) in any of the tumor samples tested.

DISCUSSION

Global gene expression profiling has been used for molecular classification of several cancers, including breast, prostate, colon, non-small cell lung cancers, lung adenocarcinoma, medulloblastoma, and melanoma, as well as of large B-cell lymphoma (38–43). This classification has been shown to correlate with clinical phenotype as defined by tumor progression and survival. Regarding bladder cancer, only limited data on gene expression profiles are available. The group of Ørntoft applied hierarchical cluster analysis to pooled suspensions of bladder cancer cells and identified gene clusters covarying with stage and grade (44). Using a cross-validation approach, this group proposed a 32-gene molecular classifier predicting disease progression of Ta tumors (45).

In this study, microarray analysis was used to identify distinct gene expression profiles of superficial, muscle-invasive, and metastasizing transitional cell carcinoma of the bladder. To clearly define the phenotype of a given tumor, only patients with uniform treatment (transurethral resection for superficial and radical cystectomy for invasive bladder cancer) and long-term follow-up were studied. Using unsupervised hierarchical cluster analysis, a clear cut separation of superficial and invasive tumors was observed. This separation was remarkably stable using different subsets of expression data. Furthermore, several gene clusters related to superficial or invasive bladder cancer were observed. For example, an up-regulation of certain genes encoding transcription factors, factors involved in protein synthesis and metabolism, as well as some proteins controlling cell differentiation, is evident.

| Table 3 Correlation of Clontech/Affymetrix expression data with real-time PCR derived values\(^{ab}\) |
|---------------------------------|-----------------|-------------------------------|---------------------|-----------------|-----------------|-----------------|
| **Gene**                        | **Array platform** | **Clontech/Affymetrix probe set** | **GenBank accession no.** | **Pearson correlation** | **r**            | **p**            |
| Decorin                         | Clontech         | D11b                          | M14219                | −0.8117             | 9.65E-07         |
| Decorin, variant A              | Affymetrix       | 201893.x.at                   | AF1383.001.1          | −0.9473             | 3.01E-04         |
| Decorin, variant D              | Affymetrix       | 211813.x.at                   | AF1383.031.1          | −0.9288             | 8.12E-04         |
| Decorin, variant C              | Affymetrix       | 211896.s.at                   | AF1383.021.1          | −0.9285             | 8.24E-04         |
| Tenascin C (cytotactin)         | Affymetrix       | 201645.at                     |                       | −0.9751             | 2.39E-05         |
| Actin, α 2                     | Affymetrix       | 200974.at                     | NM_002160.1           | −0.9707             | 4.15E-05         |
| Filamin A, α                   | Affymetrix       | 200859.x.at                   | NM_001613.1           | −0.9108             | 1.69E-03         |
| Myosin, heavy chain polypeptide 11 | Affymetrix       | 201497.x.at                   | NM_022844.1           | −0.9789             | 1.36E-05         |
| Calponin 1                     | Affymetrix       | 203951.at                     | NM_001299.1           | −0.9674             | 5.99E-05         |
| WAF1/p21                       | Clontech         | A091                          | U09579                | 0.0915              | 0.6702           |
| WAF1/p21                       | Affymetrix       | 202284.s.at                   | NM_000389.1           | 0.1556              | 0.7350           |
| STAT-induced STAT inhibitor 3  | Clontech         | B121                          | AB004904              | −0.2769             | 0.1891           |
| STAT inhibitor 3 (SOCS3)\(^{b}\) | Clontech         | B03m                          | AF029082              | −0.9252             | 3.38E-11         |
| 14-3-3 protein σ; stratifin    | Clontech         | 209260.at                     | BC000329.1            | −0.9848             | 4.43E-06         |
| IFN-induced transmembrane protein 9–27 | Affymetrix | 1) 201601.x.at 1) 1) 201601.x.at 1) 2) 214022.s.at 2) 214022.x.at | AA749101               | −0.9615             | 1.05E-04         |
| IFN-induced transmembrane protein 9–27 | Clontech | A01h                          | J04164                | −0.6791             | 2.23E-04         |

\(^{a}\) Correlations significantly positive for 9 of 11 genes.

\(^{b}\) There are three probe sets for the STAT-induced STAT inhibitor 3 on the Affymetrix GeneChip (206359.at; 206360.s.at; 214105.at). All probe sets did not detect any expression of this gene.
growth and differentiation were characteristic of superficial tumors. Similar observations were reported by the group of Ørntoft (46) who correlated gene expression patterns with stage and grade but not clinical outcome.

The dendrogram obtained by cluster analysis also identified a group of patients with muscle-invasive disease who are at high risk for bladder cancer recurrence after radical cystectomy. This was even more evident for those with organ-confined disease: while all of the poor risk branch of patients recurred this occurred only in 1 of 5 patients in the good-risk group. However, this data need to be confirmed in a larger cohort of patients.

Bladder cancer frequently occurs as multifocal disease with several simultaneous tumors scattered over the bladder. In this study, multifocal superficial bladder cancers always clustered together, suggesting a common genetic background. We cannot eliminate the possibility that this relationship is based on the genotype of the respective patient. However, clonality of multifocal bladder cancer has also been reported by several other studies based on loss of heterozygosity analysis (7, 8).

Invasive bladder tumors are histologically complex tissues consisting of a variety of cell types besides carcinoma cells. As with other methods, measuring tissue RNA expression microarrays determine expression levels irrespective of cell type. However, tumor development and progression, e.g., growth, angiogenesis, tumor cell detachment, and invasion, is dependent on interactions between tumor cells and the host microenvironment (stroma). Thus, expression of factors involved in tumor-cell stroma interactions might reflect biological properties independent of cellular origin (47–51). Although microdissection would allow the examination of highly pure tumor cell populations of a given specimen, it might be of little use in a clinical setting. Thus, identification of a molecular classifier gene set, which is to some degree independent of tumor tissue heterogeneity, is required.

To determine the influence of different tumor-stroma relations and tumor heterogeneity, specimens from different areas of invasive tumors, e.g., infiltrating, superficial, or deeper layers of the bladder wall, were included in the cluster analysis. With one exception (P36.3), all samples from the same tumor were grouped together on the dendrogram (Fig. 2B), suggesting that each part of the tumor was representative for the whole, based on multiple gene expression analysis. Notably, the only exception was found to contain the lowest proportion of tumor cells (data not shown). The clonality of different parts of invasive tumors does, however, not reflect the genetic identity of a given patient because corresponding mucosa samples appeared on a separate branch of the dendrogram.

Expression profiles of all histologically verified normal mucosa specimens, derived during cystectomy for invasive bladder cancer, were clustered together on the invasive branch of the sample dendrogram; the respective (sub-) branch contained several good-risk patients. This is in line with other reports of genetic alterations (e.g., LOH on chromosome 9 loci) in normal mucosa of bladder cancer patients, suggesting a field defect (49, 50). However, our observation that these mucosa
samples cluster into the invasive, but not the superficial (or an own distinct) branch, suggests that this mucosa is genotypically determined not only as malignant but also as potentially invasive. This raises the intriguing possibility that a gene expression profile of bladder mucosa may exist that characterizes patients with a high risk of developing invasive disease.

Because the major focus of this study was the identification of differentially expressed genes, we aimed at independent confirmation of the whole data set. Therefore, we compared gene expression data obtained by the cDNA nylon array (Atlas; Clontech) with those from an oligonucleotide GeneChip (Affymetrix). The direct comparison revealed a limited agreement between the two different array platforms (Fig. 3). A lack of agreement between gene expression data from different commercial microarray platforms has also been reported by other groups emphasizing that independent confirmation of complete gene sets for molecular classification is required (52–58).

In this study, Clontech expression data were confirmed for 117 of 270 significantly expressed genes by GeneChip analysis. Of these, 41 were significantly differentially expressed between superficial and invasive tumors on both array platforms. Thus, a set of 41 validated genes is provided, which using unsupervised hierarchical cluster analysis, resulted in a clear separation of superficial and invasive bladder cancers and defined a subgroup of tumors with adverse clinicopathological features (Fig. 4).

In summary, we used hierarchical cluster analysis on tumor specimens of a cohort of uniformly treated patients with well-defined clinical outcome. Distinct gene expression profiles of superficial and invasive tumors were observed. Furthermore, we could identify distinct prognostic groups of invasive bladder cancer. The pattern of gene and tumor clusters was remarkably stable using different subsets of gene expression data, including a set of 41 genes, which was validated using two different array platforms. We also observed (a) a clonal origin of superficial tumors, (b) highly similar gene expression patterns in different areas of invasive tumors, and (c) an invasive type of pattern in bladder mucosa derived from patients with locally advanced disease. Our data provide additional insight into the molecular pathogenesis of bladder cancer offering the opportunity to detect novel prognostic markers for superficial, invasive, and metastasizing disease.

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Identifying Superficial, Muscle-Invasive, and Metastasizing Transitional Cell Carcinoma of the Bladder: Use of cDNA Array Analysis of Gene Expression Profiles

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