Molecular Profiling of Bladder Cancer Using cDNA Microarrays: Defining Histogenesis and Biological Phenotypes

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ABSTRACT

This study was designed to characterize the expression profiles of nine bladder cancer cell lines (T24, J82, 5637, HT1376, RT4, SCaBER, TCCSUP, UMUC-3, and HT1197) using cDNA microarrays (8976 genes and expressed sequence tags). Novel targets involved in bladder cancer progression of potential clinical relevance were validated by immunohistochemistry using tissue microarrays of primary bladder tumors (n = 193 cases). Hierarchical clustering classified uroepithelial cells based on their histopathogenesis and cell cycle alterations. Keratin 10 and caveolin-1 transcripts were more abundant in tumor cells from squamous and invasive origin. Their combined expression was shown to stratify bladder tumors and define squamous differentiation. To assess the robustness of the clustering analysis, a bootstrap resampling technique was used. This grouped tumor cell lines based on their biological properties, including cell cycle and cell adhesion features. E-cadherin, zyxin, and moesin were identified as genes differentially expressed in these clusters and related to the p53, RB, and INK4A status of the cell lines. Loss of these adhesion molecules was associated with stage and grade in primary tumors (P < 0.05), and moesin expression was also associated with survival (P = 0.01). Deregulation of cell cycle and apoptotic pathways, such as mutations or altered expression of p53, pRB, and INK4A (p16), is necessary for uroepithelial transformation. However, it appears that deregulation of cell adhesion is a common event associated with tumor progression in uroepithelial neoplasms.

INTRODUCTION

Bladder cancer is one of the most common malignancies in developed countries, ranking as the sixth most frequent neoplasm (1). BBC3 is the most common malignant neoplasm in Egypt and also occurs with a high incidence in other regions of the Middle East and East Africa (2). Certain clinical and pathological features of BBC are different from those described for cTCC, such as the high incidence of detecting squamous metaplasia and the development of SCC (2). TCC has been classified into two groups with distinct behavior and different molecular profiles: (a) low-grade tumors (which are always papillary and usually superficial); and (b) high-grade tumors (which are either papillary or nonpapillary and are often invasive; Ref. 3). The inactivation of both RB and p53 pathways has been shown to be necessary for uroepithelial transformation. However, it appears that deregulation of cell adhesion is a common event associated with tumor progression in uroepithelial neoplasms.

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2 The abbreviations used are: BBC, bilateral bladder cancer; SCC, transitional cell carcinoma; cTCC, conventional TCC; SCC, squamous cell carcinoma; S-BBC, squamous BBC; T-BBC, transitional BBC; CI, confidence interval.

Received 8/9/02; accepted 9/27/02.

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equivlant to averaging the log intensity). The curvature in the scatter plot indicated a dependence on the ratio $R$ on the overall intensity. This curve is then used to normalize the data: log(red/green) = $\log(\text{red/green}) - c(A)$ where $c(A)$ is the fit. This is equivalent to multiplying the green channel intensity (or dividing the red) by an intensity dependent normalization constant $k(A)$ where $\log(k(A)) = c(A)$. Optimal normalized data should be horizontal and centered around zero. Samples were normalized using this intensity-dependent normalization using the Splus function lowess (18). Normalized fold changes in gene expression were then used to further analyze and cluster the various cell lines.

Cutoffs. The following filter values were used: the absolute value of the fold change ($R/G$ and $G/R$) had to be greater than 2.0 in at least one experiment, and the average intensity ($A$) had to be greater than 300. This filter reduced the number of genes from 8976 to 234. Data were filtered to select genes that had both a fold change to remove the background of mostly unchanging genes and an average intensity distinguishable from the noise of the microchip hybridization.

Clustering. The relationship among cell lines was analyzed using hierarchical clustering limiting to overexpressed genes with $R/G$ or $G/R$ ratios higher than 2.0 in at least one experiment (17). To assess the robustness of the clustering analysis, a bootstrap resampling technique was used to generate 1000 copies of the data set by adding Gaussian noise to the original data. The mean value of the noise was zero, and the SD was dependent on the average intensity of a given spot. To determine this intensity-dependent noise value, we used the data from a sample replicated eight times. By fitting a curve to the scatter of the SD of the eight replicates as a function of average log intensity, we obtained a curve for the average noise as a function of intensity. This was used in the Monte Carlo resampling to set the value of the SD. Each of the 1000 bootstrap samples was then clustered using the hierarchical method with the dot plot product (angle) metric and Ward linkage. A consensus tree was constructed using the CONSENS program (version 3.5c). A program constructs a tree by finding for each node the pairing that occurred most often in the 1000 separate trials. A graph was constructed that displays this count at each node of the tree. Nodes with values closer to 1000 are more robust than with lower ones.

Validation of the Results
Northern Blotting. Northern hybridization was performed using 10 µg of total RNA from the bladder cancer cell lines used in the analysis (see above) and probes generated from the cDNA clones (data not shown).

Tissue Samples and Tissue Microarrays. Three different bladder cancer microarrays were constructed for this study. Normal and tumor tissues were embedded in paraffin, and 5-µm sections were stained with H&E to identify viable, morphologically representative areas of the specimen from which needle core samples were taken, using a precision instrument (Beecher Instruments, Silver Spring, MD; Ref. 19). From each specimen, triplicate tissue cores with diameters of 0.6 mm were punched and arrayed on the recipient paraffin block. Five-µm sections of these tissue array blocks were cut and placed on charged polylysine-coated slides and used for immunohistochemical analysis. Arrayed normal tissues known to express the antigens under study were used as baseline positive controls and showed physiological expression patterns of these markers.

These tissue microarrays included a total of 173 bladder primary TCC tumors obtained under Institutional Review Board-approved protocols. Tumor stage and grade were defined according to consensus criteria (20, 21). A total of 40 superficial and 64 invasive TCC tumors were analyzed in two microarrays. These tumors corresponded to 14 grade 1, 8 grade 2, and 82 grade 3 lesions. Another tissue microarray comprised a cohort of 69 bladder primary TCC cases with known p53, p16, and pRB status, and consisted of 2 superficial and 67 invasive lesions. In addition, 20 cases of invasive BCC were also analyzed, including 14 S-BBCs and 6 T-BBCs, for a total of 193 cases. These BCC lesions were also analyzed for patterns of p53 expression (see below).

Immunohistochemistry. Protein patterns of expression of identified targets were assessed at the microanatomical level for cavelin-1, keratin 10, E-cadherin, zyxin, and moesin, using both cytopsin from all cell lines studied and tissue samples outlined above. Standard immunoperoxidase procedures were used for immunohistochemistry (19). We used the following antibodies at certain conditions: (a) cavelin-1, mouse monoclonal IgG1 at 1:1000 dilution (2.5 µg/ml; BD Transduction Laboratories, Lexington, KY); (b) keratin 10, mouse monoclonal clone DC-K10 at 1:2000 dilution (1.0 µg/ml; Neomarkers, Fremont, CA); (c) E-cadherin, mouse monoclonal clone 36 at 1:1000 dilution (2.5 µg/ml; BD Transduction Laboratories); (d) moesin, mouse monoclonal clone 38/87 at 1:50 dilution (4 µg/ml) with microwave pretreatment of the slides (Neomarkers); (e) zyxin, mouse monoclonal clone 21 at 1:25 dilution (10 µg/ml) with microwave pretreatment of the slides (BD Transduction Laboratories); (f) total RB, mouse monoclonal clone 3C8 at a final concentration of 1.2 µg/ml (QED Bioscience, San Diego, CA); (g) p16, mouse monoclonal clone DCS-50.1/H4 at 2.5 µg/ml (Calbiochem, Cambridge, MA); and (h) a mouse antihuman monoclonal antibody against p53 (1:500 dilution; Ab-2; clone 1801; Calbiochem). Staining conditions were optimized on sections from formalin-fixed, paraffin-embedded tissue controls for each antibody as specified by the manufacturers. Antibody reactivity was detected by using diaminobenzidine as chromogen, and sections were counterstained with hematoxylin. The primary antibody was omitted for negative controls. p53 staining was defined as negative (undetectable levels to ≤20% of tumor cells displaying nuclear staining) or positive (moderate to intense nuclear immunoreactivities in ≥20% of cells; Ref. 22). There is no consensus on the cutoffs of the immunohistochemical expression of the other markers, and thus they were analyzed as continuous variables or taking the cutoff of 0% versus higher than 0% when they were considered as categorical.

Data Analysis. All cTCCs ($n = 173$) were used for the analysis of association between p53 and pRB with keratin 10, cavelin-1, E-cadherin, zyxin, and moesin. These cases were also used to evaluate marker expression versus histopathological stage and tumor grade using the nonparametric Wilcoxon-Mann-Whitney and Kruskall-Wallis tests (23). The consensus value of the three representative cores from each tumor sample arrayed was used for statistical analyses. The association of keratin 10 and cavelin-1 with squamous differentiation was analyzed using the total cohort of 193 cases, including the 20 BCC tumors. Expression values will be displayed as mean values accompanied by 95% CIs and/or range.

The relationship of marker to outcome was evaluated using a subset of 69 cTCC cases for whom follow-up was available. Overall survival time was defined as the number of months elapsed between transurethral resection (two superficial lesions) or cystectomy (rest of cases) and death from disease (or the last follow-up date). Patients who were alive at the last follow-up or lost to follow-up were censored. For survival analysis, expression marker results were analyzed as continuous variables. Membrane expression of moesin was also considered as a categorical variable because its median expression value was zero. The association of the marker expression levels with overall survival was analyzed using the Wald test, and the log-rank test was used to examine their relationship when different cutoffs were applied (24). Survival curves were plotted using standard Kaplan-Meier methodology (25). Additionally, it was possible to evaluate the association of the markers with the p53 (mutation analysis) and p16 (mutation and polymorphism analysis) status in this subset of 69 patients. Associations between markers were analyzed using Kendall’s tau test.

RESULTS
Experimental Design. Two major sets of experiments were conducted. Initially, we used bladder cancer cell lines and cDNA microarrays to identify differentially expressed genes between distinct histopathological tumor types and stages of the disease. In a second approach, tissue microarrays were used to validate the potential clinical significance of the targets identified by cDNA microarrays at the microanatomical detail using immunohistochemistry on clinical material. A cohort of superficial and invasive bladder neoplasms was used to evaluate the association between molecular targets and histopathological variables including stage and grade. An additional tissue microarray, containing bladder tumors with characterized p53 alterations and annotated follow-up, was used to delineate associations between molecular markers and these critical pathways, as well as with clinical outcome.
Histopathogenetic Categorization of Bladder Cancer Cell Lines. Hierarchical clustering of cDNA microarray experiments, based on 234 genes that showed a R/G or G/R fold ratio higher than 2 and intensities higher than 300, classified these tumor cells according to the histopathological characteristics from the tumors they were obtained from. SCAberg cells, derived from a squamous carcinoma of the bladder, were distinguished from cells derived from transitional carcinomas. Moreover, tumor cells from invasive lesions clustered together and were separated from those cells derived from metastatic (TCCSUP) or superficial (RT4) bladder cancers (Fig. 1). The complete list of 234 genes will be available in the web site that is being created for this manuscript.

Caveolin-1 and Keratin 10 Are Markers of Squamous Differentiation and Are Associated with Tumor Stage and Grade. We observed that both caveolin-1 and keratin 10 were differentially expressed among the various bladder tumor cell lines analyzed. High levels of caveolin-1 were detected in SCAberg cells, whereas caveolin-1 expression was low to undetectable in RT4 cells. The expression of keratin 10 was high in several cell lines, and a previous report linked its expression to squamous bladder carcinoma (26).

Based on these observations and the availability of well-characterized antibodies to their encoded products, we further explored their patterns of expression in several normal samples of human urothelium, urothelial squamous metaplasia, and the above-mentioned tissue collections, including 173 cTCCs and 20 BBCs (16 S-BBCs and 4 T-BBCs). We found that normal urothelium and superficial cTCC had undetectable levels of both caveolin-1 and keratin 10. However, areas of squamous metaplasia and carcinoma identified in BBC as well as areas of squamous differentiation identified in cTCC, had significant expression of both proteins. Caveolin-1 was expressed in 12 of 16 of S-BBCs, as well as in 2 of 4 of T-BBCs and 72 of 173 of cTCCs, whereas keratin 10 was found in 8 of 16 of S-BBCs, 1 of 4 of T-BBCs, and 28 of 173 of cTCCs (Fig. 2). Statistical analysis of data revealed that both caveolin-1 and keratin 10 were significantly associated with identification of squamous differentiation in 49 of 193 patients (P < 0.001).

Separate analysis of the 173 cTCC lesions revealed that only 1 of 42 superficial lesions displayed caveolin-1 in few tumor cells (approximately 3% of tumor cells), whereas 70 of 131 invasive tumors showed caveolin-1 expression in 3–83% of tumor cells. Keratin 10 was undetectable in all superficial lesions, whereas 28 of 131 invasive tumors expressed keratin 10 in 3–70% of tumor cells. All grade 1 lesions showed undetectable expression of caveolin-1 and keratin 10. Grade 2 tumors expressed caveolin-1 in <3% of tumor cells, whereas in grade 3 tumors, the mean number of cells showing positive expression of caveolin-1 was 13% (95% CI, 8.9–17.2%). Keratin 10 expression was also undetectable in grade 2 tumors, and for grade 3 tumors, the mean number of cells showing positive expression of keratin 10 was 2.3% (95% CI, 0.5–4.1%). Overall, there was a statistical association between caveolin-1 expression and both tumor stage (P < 0.001) and grade (P < 0.001). Keratin 10 also reached a significant statistical association with tumor stage (P = 0.019) and grade (P = 0.018).

We postulate that cTCCs in which these products were identified harbor morphologically unrecognized areas of squamous differentiation. This could have important clinical implications because it has been reported that invasive bladder tumors with squamous features do not respond to methotrexate, vinblastine, adriamycin, and cisplatin (MVAC) treatment (see “Discussion”).

Discussion.

Clustering Associates Expression Profiling with Biological Phenotypes Related to p53 and RB Pathways. A Monte Carlo bootstrap method was applied to establish the robustness among the grouping of the cell lines based on the expression of 234 genes selected from the cDNA microarrays showing a R/G or a G/R fold ratio higher than 2 and intensities higher than 300. Using this approach, we observed that the analyzed tumor cells assembled based on their reported molecular alterations related to the p53 and RB signaling pathways (Fig. 3). Two main clusters or groups were identified: (a) T24, SCAberg, and UMUC-3 (group 1); and (b) HT11376, HT1197, and TCCSUP (group 2). Briefly, cells that harbor TP53 mutations at exons 4 and 5, detectable pRB, and INK4A mutations (group 1) clustered together and were distinguishable from those with TP53 mutations affecting exons 7, 10, and 11; undetectable levels of pRB; and a wild-type INK4A locus (group 2; Ref. 7). Cells with p53 mutations in exon 8 showed a higher distance from this second group. In an attempt to identify genes related to this clustering and of potential biological significance, a search was performed for common over- and under-expressed genes unique to each cluster group. Of interest, only three known genes (zyxin, protocadherin 13, and moesin) and an expressed sequence tag were found differentially expressed between the two significant clusters. To validate these results at the protein level, immunohistochemical studies using antibodies to zyxin, moesin, and E-cadherin [a downstream product of protocadherin 13 (27) for which antibodies are not available] were performed on cytopsins from the analyzed bladder cancer cell lines. Cells from group 1 displayed lower transcript levels of protocadherin 13 and zyxin than cells from group 2. Alternatively, group 2 cells had lower transcript levels of moesin than cells from group 1. Immunohistochemical phenotyping of these cell lines using antibodies to E-cadherin, zyxin, and moesin confirmed the above-mentioned results and are shown on the web site.

Zyxin, E-Cadherin, and Moesin Are Associated with Altered pRB Expression, TP53 Mutation Localization, and Tumor Stage and Grade in Primary Bladder Cancer. To validate the results obtained through permutation clustering using clinical primary bladder tumors, patterns of p53 and pRB expression were assessed in the cohort of patients under study. In addition, TP53 mutation status was established in a subset of 69 cTCC cases: 37 tumors had wild-type TP53; 9 lesions harbored mutations affecting exons 4 or 5; and 23 tumors had a mutation between exons 6 and 11 (28). Cytoplasmic zyxin expression was significantly associated with detection of TP53 mutations affecting exons 6–11 (P = 0.03). With regard to p53 and
pRB expression status: 73 cases displayed a p53-positive phenotype [nuclear immunoreactivities in ≥20% tumor cells (22)], whereas the remaining cases were classified as having a p53-negative phenotype; 44 cases had undetectable pRB, whereas the remaining cases showed heterogeneous pRB nuclear immunoreactivities. Zyxin was observed in the cytoplasm of 102 of 173 cTCC cases, whereas membrane E-cadherin staining was found in 146 of these 173 lesions (Fig. 4). Moesin was detected as a membrane staining in 26 of 173 cTCCs (Fig. 4). No statistical association was found between expression patterns of p53 and either zyxin, E-cadherin, or moesin. Nor did we find any relationship among mutations, polymorphisms, or protein overexpression of p16 in a subset of 69 patients for whom INK4a/p16 was available. However, we observed mutation in codons 36, 156, 245, and 285 of TP53, and these four cases died of the disease.

**DISCUSSION**

Data from this study provide new insights regarding critical issues in bladder cancer. Expression profiling classified the nine bladder

**Moesin Is a Predictive Marker in Bladder Cancer.** The potential overall survival prognostic utility of caveolin-1, keratin 10, E-cadherin, moesin, and zyxin was evaluated using 69 cTCCs for which clinical follow-up was available. Membrane moesin expression was associated with overall survival (P = 0.01) in this subset of 69 patients (Fig. 5). We observed that patients presenting a positive moesin expression displayed p53 ≥ 20% in 5 of 10 cases, E-cadherin > 0% in 6 of 10 cases, zyxin > 0% in 6 of 10 cases, total pRb ≥ 10% in 9 of 10 cases, underphosphorylated pRb ≥ 10% in 3 of 10 cases, and p16 > 0% in 2 of 10 cases. No polymorphism or mutation in the INK4a/p16 gene was detected. However, we observed mutation in codons 36, 156, 245, and 285 of TP53, and these four cases died of the disease.
...cancer cell lines under study based on the histopathological characteristics of the tumors from which they were obtained. We found that keratin 10 and caveolin-1 expression was associated with the presence of squamous differentiation, as well as with pathological stage and tumor grade. The application of bootstrapping techniques to hierarchical clustering grouped most of the bladder cancer cell lines based on their alterations in p53 and RB pathways. This finding is relevant because both p53 and RB have been reported to be involved in bladder cancer progression. Moreover, the identified target genes, zyxin, E-cadherin and moesin, were associated with p53 and/or pRB alterations in the primary bladder tumors analyzed. Furthermore, they were also significantly associated with bladder cancer progression, and moesin provided predictive outcome information. As a corollary to the studies conducted, it should be noted that identified target genes obtained from high-throughput molecular profiling of cultured cells were also shown to have clinical impact when validated in primary bladder tumors using tissue microarrays.

Bladder cancer comprises a variety of distinct neoplastic disorders. Transitional and squamous carcinomas are the most prevalent forms of bladder cancer. However, adenocarcinomas, small cell tumors, and neuroendocrine tumors are also found as primary bladder tumors with a lower frequency. Identification of the prevailing and, if present, secondary histogenetic features of the tumor has significant clinical connotations because the lack of response to certain therapeutic regimens in the context of specific tumor types is well known. For example, squamous carcinoma of the bladder has been reported to be more resistant to radio- and chemotherapy than conventional transitional bladder tumors (26). Data from this study revealed a characteristic pattern of caveolin-1 and keratin 10 expression in early squamous metaplasia and squamous carcinomas in the setting of BBC. In addition, we observed that the expression of caveolin-1 and keratin 10 in certain cTCCs, usually identified as clusters of tumor cells heterogeneously stained within the bulk of the tumor. Furthermore, there was a significant association regarding detection of both caveolin-1 and keratin 10 in the bladder tumor samples analyzed. Thus, they may serve as markers of squamous differentiation before the morphological identification of this cellular phenotype. This phenomenon might be linked to the lower response to MVAC observed in certain cTCCs harboring histologically unrecognizable areas of squamous differentiation and may be of assistance in selecting those patients that would benefit from other therapeutic regimens (26). Keratin 10 is recognized as a marker of squamous differentiation, and its expression has been observed previously in squamous bladder carcinomas (26, 29). The increased expression of caveolin-1 has been related to cellular transformation and tumor progression (30) and is associated with augmented cell signaling activity. One of the molecules that is organized and concentrated in the scaffolding domain of caveolin-1 is the epidermal growth factor receptor (30). Bladder cancer cells have increased growth factor receptors, including epidermal growth factor receptor and Her-2/Neu proteins (31), and this phenomenon has been associated with tumor progression. Interestingly, data from the present analysis also link caveolin-1 expression with increased tumor pathological stage and tumor grade. A recent study has also described association with tumor grade but not with patient outcome (32). The extent to which keratinization and the role of caveolin-1 as a membrane protein implicated in selective transcytosis and increased signaling are critical in bladder cancer progression and chemoresistance in tumors presenting squamous differentiation has to be further studied.

One of the most notable findings of this study is that significant clusters obtained by bootstrapping methods grouped the bladder cancer cell lines analyzed based on their p53 and RB pathway status. Growth control in mammalian cells is accomplished largely by the action of pRB, which regulates exit from G1, and the p53 protein, which triggers growth arrest or apoptotic processes in response to cellular stress. In tumorigenesis, pRB and p53 serve collaborative roles, as evidenced by their frequent alterations in human tumors, including bladder cancer (6, 7, 33–35). The mechanistic basis for this dual requirement stems, in part, from the deactivation of a p53-dependent cell suicide program that would normally be brought about as a response to unchecked cellular proliferation resulting from RB deficiency. Two significant major patterns arise from the bootstrapping analysis study in these cell lines based on the combined expression of 234 genes. We contrasted these groups with the molecular characterization of p53/pRB/INK4a in these cells lines described previously (7). We observed that the combined alteration of these critical networks could support these clusters. Cells harboring TP53 mutations in the NH2-terminal transactivation domain, presenting with high pRB levels and INK4A mutations grouped together (UMUC-3, SCaBER, and T24), and certain cells with TP53 mutations in the core domain, undetectable pRB levels, and wild-type INK4A locus were in the same cluster (HT1197, HT1376, and TCCSUP). TP53 mutations in the core domain affect the ability of p53 to bind DNA and are associated with loss of the contralateral allele (35), completely inactivating p53 function and thus impacting both cell cycle arrest and induction of apoptosis. However, mutations in the transactivation domain result in products that preserve p53 activities, such as DNA binding, to some extent. This incomplete p53 suppressive phenotype is usually associated with other alterations in the pathway, mainly p14ARF mutations or Hdm2 amplification/overexpression. In these cases, we found detectable pRB expression, but the other prevalent mutation of the RB pathway, namely, p16/INK4A deletions, was detected in all cell lines displaying such a genotype. Finally, we observed that RT4 cells had a wild-type TP53 but harbored a homozygous INK4A deletion and lacked pRB expression. These data support the working model reported previously (7). How-

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>RT4</th>
<th>UMUC3</th>
<th>J82</th>
<th>T4</th>
<th>SCaBER</th>
<th>3607</th>
<th>TCCSUP</th>
<th>HT1197</th>
<th>HT1376</th>
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<td>-0.083</td>
<td>-1.233</td>
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<tr>
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<td>1.021</td>
<td>0.733</td>
<td>-0.549</td>
<td>-0.607</td>
<td>-0.796</td>
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Fig. 3. To assess the robustness of the clustering analysis, a bootstrap resampling technique was applied. First, a large number (1000 in this analysis) of copies of the data are generated using a Monte Carlo resampling technique. Each of these generated data sets is then clustered using the standard hierarchical method; in this case with the dot product (angle) metric and ward linkage. The 1000 resulting trees are then used to build a consensus tree using the CONSENS program from the PHYLIB package. The output consists of a count at each node of the tree that represents how many of the 1000 trees had that bipartition. Nodes with values close to 1000 are more significant than others scoring lower values. The higher the number at each node of the tree, the more similar the expression patterns of the cells within clusters are. Tumor cell lines displaying similar alterations in the TP53, RB, and ARF/p16 pathways grouped together within significant clusters. In the bottom part of the figure, the logarithmic ratio of the expression of these three genes in each of the cell lines is shown. Positive and negative numbers mean higher and lower expression of these genes, respectively, among the cells.
ever, we also observed that cells known to harbor mutations in exon 8 in the core domain (5637 and J82) were not included in the cluster described by the bootstrap technique. Although we believe that p53/PRB/INK4A pathways are critical in bladder cancer, these clusters are based on 234 genes, and further investigation is warranted to identify other networks involved in the development and progression of bladder cancer.

Three adhesion-related molecules, zyxin, E-cadherin, and moesin, were found to be associated with the p53/RB patterns discussed above. Those cell lines harboring TP53 mutations in the core domain and lacking pRB displayed low moesin transcript levels, whereas those cell lines with TP53 mutations in the transactivating domain and high levels of pRB displayed low zyxin and E-cadherin. Moreover, the associations observed in cell lines were also found and validated in primary bladder tumors. Low levels of moesin, zyxin, and E-cadherin were significantly associated with advanced pathological stage and higher tumor grade, supporting their involvement in bladder cancer progression.

Alterations of E-cadherin have been described as common events in bladder cancer (10, 11). Our observations regarding the association of E-cadherin with histopathological stage and tumor grade were in accordance with other previous studies (10, 11). However, we did not find a significant association with outcome, probably due to the number of patients analyzed. Loss of zyxin has been associated with neoplastic transformation because it was found as a marker of acute myeloid leukemia subtype (13). The functional role of zyxin is not

<table>
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<tr>
<th>Table 1 Association between expression levels of moesin, E-cadherin, and zyxin with expression levels of pRB</th>
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<tbody>
<tr>
<td>Zyxin expression was also significantly associated with TP53 mutation sites.</td>
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<td>No. of cases</td>
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<td>Association with RB expression</td>
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<tr>
<td>Moesin</td>
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<tr>
<td>E-Cadherin</td>
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<td>Zyxin</td>
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<td>Association with TP53 mutation</td>
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<td>Zyxin</td>
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Fig. 4. Representative immunostaining patterns of zyxin, moesin, and E-cadherin in primary bladder tumors. Superficial tumors showed high levels of E-cadherin (A), zyxin (B), and moesin (C) expression. However, invasive bladder neoplasms were found to express low to undetectable levels of these proteins [E-cadherin (D), zyxin (E), and moesin (F)]. There was a significant difference between the expression of these proteins and histopathological stage and tumor grade ($P < 0.0005$). Original magnifications: A–C, ×200; D–F, ×400.
The three adhesion-related markers (zyxin, E-cadherin, and moesin) found differentially expressed in bladder cancer in our study share a common feature: a relationship to the β-catenin pathway. β-Catenin is a cytoplasmic protein that participates in the assembly of cell-cell adhesions junctions by binding cadherins to the actin cytoskeleton. The cytoplasmic domain of E-cadherin interacts directly with β-catenin. Zyxin is a cytoplasmic adhesion junction protein found in complexes with α-actinin and actin (40). The association of zyxin with cadherins has also been reported previously (41). Moesin is a member of the ERM (ezrin, radixin, and moesin) family of proteins located just beneath the plasma membranes, which are also thought to be involved in the association of actin filaments with the plasma membrane regulating cell-cell and cell-matrix adhesion (42). The association of ezrin with E-cadherin and β-catenin has also been revealed by coprecipitation studies (43). The involvement of β-catenin in bladder cancer progression has recently been described in murine and human models (43), an observation supporting our findings. Among downstream targets of β-catenin (44–46), cyclin D1 has been shown to be critical in G1-S cell cycle transition by phosphorylating pRB. Interestingly, the three identified markers were associated with RB gene expression in primary tumors, and this association could be attributed to alteration of cyclin D1 expression levels as reported in colorectal and desmoid tumors (46).

Disruption of the β-catenin signaling pathway by alterations in the physiological balance between its interactions with zyxin, E-cadherin, or moesin could mechanistically account for the invasiveness potential that certain bladder cancer cell lines under study display (43). Other alternative mechanisms affecting this pathway include alterations of the Wnt signaling (44), RAS mutations (47), and mutations affecting the β-catenin gene itself (43). Aberrant accumulation of β-catenin in solid tumors has been also associated with mutational inactivation of the TP53 gene. Overexpression of wild-type p53, by either transfection or DNA damage, has been shown to down-regulate β-catenin in human and mouse cells (48). However, whether the association between zyxin and TP53 mutations could also be related to an altered β-catenin pathway remains to be elucidated.

This study has identified the deregulation of three adhesion molecular targets as common alterations in high-grade bladder cancer cells with different described phenotypes of cell cycle regulator genes. The loss of these cell adhesion molecules was correlated with tumor progression in primary bladder tumors. This observation reveals the importance of the interactions among tumor cells as well as interactions of tumor cells with the surrounding stroma in cancer progression. It appears that deregulation of cell cycle and apoptotic pathways, such as mutations or altered expression of p53 and pRB, are necessary for uroepithelial transformation (6–8). However, they appear to be insufficient for bladder cancer progression. Even though linear models are a “simplification” of complex pathological events, it appears that deregulation of cell adhesion is a common event associated with tumor progression in bladder cancer, independently of the genetic alterations triggering tumorigenesis. Expression profiling has revealed the common deregulation of cell adhesion displayed in highly invasive cells through alternative adhesion pathways (9–12). The most relevant finding was that these molecular targets identified in vitro, zyxin, E-cadherin and moesin, were found to be critical in clinical material, supporting a relevant role of deregulation of cell adhesion in bladder cancer progression.

In summary, molecular profiling using cDNA microarrays clustered bladder cancer based on both histopathogenesis and biological criteria. Novel target genes have been validated using tissue arrays con-

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**Fig. 5. Survival analysis for patients with bladder tumors stratified by moesin expression treated as a categorical variable.** The Kaplan-Meier method was used to estimate overall disease-free survival; log-rank analysis was used to compare the curves. Detection of moesin in the membrane of tumor cells from primary tumor samples was found to be significantly associated with overall survival in this subset of 67 bladder cancer patients (median follow-up time, 36 months; P = 0.01).

<table>
<thead>
<tr>
<th>Marker</th>
<th># Events</th>
<th># Censored</th>
<th>Median Survival (months)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moesin (membrane)</td>
<td>32</td>
<td>25</td>
<td>36.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Undetectable</td>
<td>7</td>
<td>3</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Detectable (≥0%)</td>
<td></td>
<td></td>
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</tbody>
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EXPRESSION PROFILING OF BLADDER CANCER

taining well-characterized primary tumors. Keratin 10 and caveolin-1 defined squamous differentiation and might become useful markers to further stratify bladder tumors. E-cadherin, moesin, and zyxin were associated with tumor progression, revealing the importance of deregulation of cell adhesion in bladder cancer progression. Finally, moesin expression appeared to be a significant prognostic factor associated with patient survival.

ACKNOWLEDGMENTS

We thank Thomas Belbin, Thomas Harris, and Yi Wei for assistance in cDNA technology and Jaya Satagopan and David Verbel for assistance in statistical analyses. We also thank Drs. Guido Dalbagni, William Gerald, and Marc Ladanyi for suggestions and critical review of the manuscript.

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