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 immunohistochchemistry 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). BBC 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 required for the transformation and immortalization of uroepithelial cells (4–7), and their alterations are common and of predictive nature in clinical studies of bladder cancer (5, 8). Cross-talk between these pathways and adhesion signaling, such as those generated by cadherin-catenin complexes, have been described to be involved in bladder cancer progression (9–12).

In the post-genome era, and in view of the advent of high-throughput methods of molecular analysis, it is expected that specific tumor types will have distinct gene expression profiles (13, 14). The elucidation of the molecular events involved in tumorigenesis and tumor progression is leading directly to the discovery and application of novel biological markers. The diagnosis and prognosis of certain neoplasms are in many cases enhanced by the use of such markers, and the marker itself may constitute a therapeutic target. In the present study, we have attempted to further characterize bladder cancer and to validate new targets involved in bladder tumor progression using a combination of cDNA and tissue microarray technologies.

MATERIALS AND METHODS

Cell Culture and RNA Extraction

Nine bladder cancer cell lines (T24, J82, 5637, HT1376, RT4, SCaBER, TCCSUP, UMUC-3, and HT1197) were obtained from American Type Culture Collection (Manassas, VA) and cultured under identical conditions following standard procedures. All cells were grown and harvested at 75–90% confluence no longer than 4–6 passages in culture for the extraction of total RNA using the RNeasy protocol (Qiagen, Valencia, CA). Cytospins were also prepared and later used for target validation.

Preparation of cDNA Microarrays

A set of 8976 sequence-verified human IMAGE cDNA clones, representing both known genes and expressed sequence tags, were PCR-amplified and spotted onto polylysine-coated microscope slides using a custom robot designed and built at Albert Einstein College of Medicine (15).

Labeling of cDNA, Hybridization to Arrays, and Image Acquisition

Ten μg of total RNA of each cell line were labeled with Cy5 (red) and hybridized against 10 μg of total RNA of a pool containing equal RNA quantities of all of these cell lines labeled with Cy3 (green). Labeling and hybridization of cDNA to arrays were carried out as described previously (16). We carried out one duplicate or one reverse-labeling experiment for validation of expression changes of the hybridization of the cell lines. After hybridization, slides were washed, dried, and scanned by a custom-built laser scanner (15). Intensity data were integrated with 8x oversampling (15). Scanalyse software was used for gridding and calculation of red (R) and green (G) signal intensities (17).

Collection and Analysis of the Data of the cDNA Microarrays

Normalization. Before any analysis, plots of the fold change versus the average intensity were examined to look for abnormalities in single-array data. It is common to plot a red versus green channel scatter plot to examine distribution of intensities; however, we found that transforming to fold change versus average intensity displayed the data in a more easily viewed form. If Ired is the background subtracted red channel intensity, and Igreen is the background subtracted green intensity, then the following variables were created: R = Ired/Igreen and A = √(Ired × Igreen), where R is simply the fold change ratio, and A is the average intensity (the geometric mean which is...
BBC lesions were also analyzed for patterns of p53 expression (see below). Analyzed, including 14 S-BBCs and 6 T-BBCs, for a total of 193 cases. These and 67 invasive lesions. In addition, 20 cases of invasive BBC were also TCC cases with known p53, p16, and pRB status, and consisted of 2 superficial rays. These tumors corresponded to 14 grade 1, 8 grade 2, and 82 grade 3 stage and grade were defined according to consensus criteria (20, 21). A total tumors obtained under Institutional Review Board-approved protocols. Tumor Arrayed normal tissues known to express the antigens under study were used. Charged polylysine-coated slides and used for immunohistochemical analysis.

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 tissue microarrays, containing bladder tumors with characterized p53 expression, 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, caveolin-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 caveolin-1 with squamous differentiation was analyzed using the total cohort of 193 cases, including the 20 BBC 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 level 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 and pRB 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. SCaBER 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.6

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 SCaBER 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 137 of cTCCs, whereas keratin 10 was found in 8 of 16 of S-BBCs, 1 of 4 of T-BBCs, and 28 of 137 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 unrecognizable 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”).

<table>
<thead>
<tr>
<th>SCaBER</th>
<th>HT1376</th>
<th>HT1197</th>
<th>J82</th>
<th>TCCSUP</th>
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<tbody>
<tr>
<td>5637</td>
<td>T24</td>
<td>UMUC3</td>
<td>RT4</td>
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Fig. 1. Hierarchical clustering of bladder cancer cell lines by overexpressed genes displaying R/G ratios higher than 2.0 in at least one experiment obtained by standard clustering analysis (17). Using this approach, tumor cell lines were classified according to histopathogenesis criteria. SCaBER cells, derived from a squamous carcinoma of the bladder, were separated from cells derived from conventional transitional carcinomas. Tumor cell lines derived from invasive tumor samples were separated from cells obtained from metastatic (TCCSUP) and superficial (RT4) bladder tumors.

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 cell classes 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, SCaBER, and UMUC-3 (group 1); and (b) HT1376, 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 cytopsin 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.6

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

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6 www.mskcc.org/GCL/BladderGenomics.
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...
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 RB 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 BC. 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-
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 fully understood,

Table 1 Association between expression levels of moesin, E-cadherin, and zyxin with expression levels of pRB

<table>
<thead>
<tr>
<th>Zyxin expression</th>
<th>No. of cases</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Association with RB expression</td>
<td>158</td>
<td>0.005</td>
</tr>
<tr>
<td>Moesin</td>
<td>153</td>
<td>0.003</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>151</td>
<td>0.001</td>
</tr>
<tr>
<td>Zyxin</td>
<td>65</td>
<td>0.03</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 < 0.005). Original magnifications: A–C, ×200; D–F, ×400.
and desmoid tumors (46). To alteration of cyclin D1 expression levels as reported in colorectal expression in primary tumors, and this association could be attributed

Interestingly, the three identified markers were associated with RB gene

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 adherens junctions by binding cadherins to the actin cytoskeleton. The cytoplasmic domain of E-cadherin interacts directly with β-catenin. Zyxin is a cytoplasmic adherens 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 down-stream 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 adscribed 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-
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.

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