Combined Gene Expression and Genomic Profiling Define Two Intrinsic Molecular Subtypes of Urothelial Carcinoma and Gene Signatures for Molecular Grading and Outcome

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Abstract

In the present investigation, we sought to refine the classification of urothelial carcinoma by combining information on gene expression, genomic, and gene mutation levels. For these purposes, we performed gene expression analysis of 144 carcinomas, and whole genome array-CGH analysis and mutation analyses of FGFR3, PIK3CA, KRAS, HRAS, NRAS, TP53, CDKN2A, and TSC1 in 103 of these cases. Hierarchical cluster analysis identified two intrinsic molecular subtypes, MS1 and MS2, which were validated and defined by the same set of genes in three independent bladder cancer data sets. The two subtypes differed with respect to gene expression and mutation profiles, as well as with the level of genomic instability. The data show that genomic instability was the most distinguishing genomic feature of MS2 tumors, and that this trait was not dependent on TP53/MDM2 alterations. By combining molecular and pathologic data, it was possible to distinguish two molecular subtypes of Ta and T1 tumors, respectively. In addition, we define gene signatures validated in two independent data sets that classify urothelial carcinoma into low-grade (G1/G2) and high-grade (G3) tumors as well as non-muscle and muscle-invasive tumors with high precisions and sensitivities, suggesting molecular grading as a relevant complement to standard pathologic grading. We also present a gene expression signature with independent prognostic effect on metastasis and disease-specific survival. We conclude that the combination of molecular and histopathologic classification systems might provide a strong improvement for bladder cancer classification and produce new insights into the development of this tumor type. Cancer Res; 70(9); 3463–72. ©2010 AACR.

Introduction

Urothelial carcinomas originate from the epithelial cells of the inner lining of the bladder wall. The majority of the tumors are papillary and confined to the urothelial mucosa (stage Ta) or to the lamina propria (stage T1), whereas the remaining are muscle-invasive (T2–T4). Most Ta tumors are of low grade, rarely progress, and are associated with a favorable prognosis, whereas high-grade Ta (T1G3) and T1 tumors represent a significant risk of tumor progression. Patients with T1 and T4 tumors are typically treated by transurethral resection; however, in spite of treatment, up to 70% of patients show local recurrences, making a regular follow-up by cystoscopy necessary. Cytogenetic studies have revealed several recurring chromosomal changes associated with tumor stage (1, 2). Traditional CGH and array-CGH have corroborated many of these findings but have also defined several recurrent high-level amplifications and deletions (3–8). The key results of these investigations are losses of chromosome 9, or 9p and 9q, frequent amplification of 6q22 (9–11), and loss of RB1 (12). Apart from chromosomal changes, several genes are known to be mutated in bladder cancer including FGFR3, PIK3CA, KRAS, HRAS, NRAS, TP53, CDKN2A, and TSC1, of which activating mutations in FGFR3 and inactivating mutations in TP53 are the most frequent. The accumulated data have shown that FGFR3 mutations are characteristic for low-grade and low-stage tumors (13), whereas TP53 mutations are characteristic for invasive tumors. This has led to the suggestion that urothelial carcinoma evolve through at least two molecular pathways, one related to FGFR3 and one related to TP53 (14). Hence, urothelial carcinomas show characteristic genetic changes both at the chromosome and at the gene level. Gene expression profiling has further increased our molecular knowledge of urothelial carcinoma (15–26).
molecular subtypes (15, 17, 20), studies have reported gene expression signatures associated with carcinoma in situ and superficial bladder carcinoma (16, 18), clinical outcome (18, 22), and progression to invasive growth (24). The behavior of individual tumors is most likely a consequence of factors operating at the chromosomal, gene, and gene expression levels. Hence, for a proper molecular understanding of urothelial carcinomas, more than one molecular level has to be considered. We therefore performed gene expression analyses of 144 cases of urothelial carcinoma representing all stages and grades, and in addition, produced genomic analyses of 144 cases of urothelial carcinoma representing all stages and grades, and in addition, produced genomic analyses of 144 cases of urothelial carcinoma representing

Materials and Methods

Tumors. Urothelial carcinomas (pathologic and clinical data are given in Supplementary Table S1) were collected by cold-cup biopsies from the exophytic part of the bladder tumor in 144 patients undergoing transurethral resection at the University Hospital of Lund, Sweden. Normal urothelial tissue was obtained from patients in surgery for non-urothelial carcinoma-related disorders. Informed consent was obtained from all patients and the study was approved by the Local Ethical Committee of Lund University.

Isolation of nucleic acids. RNA was isolated using Trizol (Invitrogen) and purified on RNeasy columns (Qiagen). RNA sample integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent) and samples with RNA integrity numbers lower than 7 were excluded. DNA from 72 of the samples was extracted using the DNeasy Tissue kit protocol (Qiagen) and from 31 of the samples using the organic phase of the Trizol lysate.

Gene expression profiling. Data from two different microarray platforms were used; a 25K cDNA array and a 35K oligonucleotide array. cDNA were labeled and hybridized to cDNA microarrays as described in Lindgren and colleagues (20). For the oligonucleotide arrays, labeling and microarray hybridization was performed using the Pronto Plus System (Promega). Arrays were scanned with an Agilent G2565AA scanner (Agilent Technologies), and quality filtered and normalized within BioArray Software Environment (27). A pin-based Lowess-fit normalization algorithm was used for normalization (28). Reporters were merged on gene symbols using a signal-to-noise–based weighted approach (29) and features with more than 20% missing values removed. To correct for platform-specific biases, e.g., differences in dynamic ranges, a set of 14 samples were hybridized to both platforms and the linear regression line for each gene present on both platforms was calculated over the 14 samples. The regression lines were then used to rescale expression values for samples hybridized to the oligo platform. Also, a correlation-cutoff was applied ($r < 0.6$). This operation removes genes with low interplatform correlations but also has a similar effect as a variance filter (Supplementary Fig. S1A and B). The robustness of the merging process was then confirmed by hierarchical cluster analysis (HCA) using an additional set of 10 samples hybridized to both platforms (Supplementary Fig. S1B). Finally, missing values were imputed when present in less than 20% of the cases using the MultiExperiment Viewer software suite ($k = 10$; ref. 30). After merging, the final data set included 144 tumors, 12 normal samples, and 2,506 high-quality genes. All microarray slides were obtained from the Sagen DNA microarray resource center (31). Data are available through the Gene Expression Omnibus (32) with accession number GSE19915.

External data sets. Data sets from Sanchez-Carbajo and colleagues (22), Stransky and colleagues (25), and Blaveri and colleagues (15) were used as external independent validation data. The Sanchez-Carbajo data were downloaded from the home page of the Journal of Clinical Oncology (33) and the Stransky data were from ArrayExpress (ref. 34; accession E-TABM-147). For both data sets, a signal filter of 300 was applied, the values in each column normalized, and the rows (genes) divided with the mean of the row to produce gene expression ratios that were converted to log 2 ratios. Reporters for the same genes were merged and genes present in <80% of the samples were removed, followed by imputation of missing values. The final Sanchez-Carbajo data set included 90 unique tumors and 4,769 genes, whereas the Stransky set had 55 unique tumors and 3,987 genes. The Blaveri data, downloaded from the home page of Clinical Cancer Research (35), were comprised of 75 tumors and 1,261 genes.

Functional analyses. The Gene Set Enrichment Analysis (GSEA) software (36) was used for interpretations of gene signatures. Genes were ranked according to their $t$ statistics and used as an input to the GSEA. We used gene sets made available through the Molecular Signatures Database (37): for E2F3, BILD_E2F3 (38), and for chromosomal instability, CARTER_GENOMIC_INST (39). $P$ values, false discovery rates, and family-wise errors of the obtained enrichment scores were estimated by permutation tests and adjusted for multiple testing. GO analyses were performed using EASE software (40).

Gene predictors. We used the GEMS algorithm (41) to construct support vector machine (SVM) predictors using 30-fold cross-validation for the stage, grade, CI/CIL, disease-specific survival (DSS) predictors, and 10-fold cross-validations for the progression predictors. We used a polynomial kernel, default values for the SVM variables, and a preselection of genes by a Kruskal-Wallis nonparametric ANOVA. We optimized the number of genes by using a preselected range of 100 to 160 and using trials with a step size of 15. The efficiency of the predictor was estimated by a receiver operator characteristic (ROC) analysis and recorded as the area under the curve (AUC). When the Sanchez-Carbajo and the Stransky data sets were used for cross-validation, pairs of data sets were constructed that included genes in both the present and the selected validation set, reducing the number of genes to 1,540 and 1,202, respectively. The SVM was then repeated as above except that a fixed number of genes (150) was used for the predictor. Our ROC-based predictor used a hold-out procedure repeated 100 times with one-third of the
cases for testing, from which average AUC was calculated for each gene. The average expression for the top 100 ranking genes with respect to absolute AUC values was selected, i.e., both genes positively and negatively correlated with the feature. A score was calculated for each case by computing the sum of expression values for genes positively correlated with the feature, minus the sum of expression values for genes negatively correlated with the feature, which subsequently was used to classify the cases into high and low scoring. The performance of this classifier was evaluated using ROC analysis. The Blaveri data were not used in these investigations due to the small number of overlapping genes ($n = 329$).

**Array CGH genomic profiling.** DNA from 103 samples were hybridized to 32K BAC arrays (CHORI BACPAC Resources; ref. 42) produced at the SWEGENE DNA Microarray Resource Center (43). BAC clones were mapped to the hg17 build (44). Array-CGH hybridizations for 36 of the samples were previously published (8). Images were analyzed using Genepix v4.0 (Axon) and loaded into BioArray Software Environment (27, 28). Flagged spots were removed and positive and nonsaturated spots were background-corrected and then log 2 ratios were calculated. The data were filtered for signal to noise ratio $\geq 5$ in both channels. Log 2 ratios on each array were normalized and corrected for intensity-dependent log ratio biases using the popLowess method (45). After normalization, log 2 ratios for each sample were segmented using a circularly binary segmentation algorithm (46) and default variables, except for SD which was set to 1 and the number of consecutive clones in each segment to be at least 4. Segmented log 2 ratios $>0.8$ were considered amplifications, $-0.2$ to $-0.8$ as deletions, and those lower than $-0.8$ as homozygous deletions. Focal amplifications and deletions were manually evaluated.

**Mutation analyses.** Genomic DNA was amplified using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare).

![Figure 1](image)

**Figure 1.** Gene expression analyses. A, HCA showing the two major gene expression subtypes of urothelial carcinomas. Green, G1 and G2; red, G3; black, nested variant or normal cases. B, heat map of the coclustering frequencies reorganized by hierarchical clustering. Red, coclustering frequency close to 1; green, coclustering frequency close to 0; black, coclustering frequency equal to 0.5. C, each tumor was scored for the mean expression levels of the genes in the MS1_UP and MS2_UP gene sets and plotted in the diagram with MS2_UP scores on the X-axis and MS1_UP on the Y-axis. Green, T0 tumors; blue, T1 tumors; red, $\geq$T2 tumors; open circles, nested or T0 tumors.
Coding regions in *FGFR3*, *PIK3CA*, *KRAS*, *HRAS*, *NRAS*, *TP53*, *CDKN2A*, and *TSC1* were selected and PCR-amplified using oligonucleotide primers (Supplementary Table S2). Reactions were sequenced using the BigDye terminator v1.1, or 3.1 cycle sequencing kit on a 3130 Genetic Analyzer (Applied Biosystems). Sequence traces were analyzed in SeqScape v2.5 (Applied Biosystems). Sequence variations were resequenced using an independent PCR.

**Statistical analysis.** Expression data were analyzed by HCA and multidimensional scaling using Euclidean distances. For HCA, 1-Pearson correlation was used as distance measure and average linkage and Wards algorithm for agglomeration. To identify stable tumor classes in the data, we used a bootstrap procedure. The cases were first clustered with HCA producing two major clusters in all four data sets. The data sets were then bootstrapped 20,000 times, analyzed by HCA, and the number of coclustering instances recorded, producing a matrix of coclustering frequencies. The resulting matrices were then analyzed with HCA as before. To identify differentially expressed genes, significance analysis of microarrays, as implemented in the MultiExperiment Viewer was used. Cox regression was used to analyze the continuous variables associated with clinical parameters and Kaplan-Meier estimators to analyze differences in clinical behavior between groups.

**Results**

**Two molecular classes of urothelial carcinoma as defined by gene expression.** The tumor cases were grouped with a 91% concordance for grade and 78% concordance for stage by the initial HCA (Fig. 1A). The lower concordance for stage was mainly caused by 23 T1 high-grade (G3) tumors clustering with the muscle-invasive (≥T2) tumors. The multidimensional scaling analysis corroborated the presence of two major tumor clusters, one densely organized composed of T4 and G1/G2 tumors, and one more heterogeneous cluster composed of ≥T1 and high-grade tumors (Supplementary Fig. S2A and B). The robustness of the HCA results was investigated by estimating the coclustering frequency using a bootstrap approach. The resulting coclustering frequency matrix was reorganized by HCA and revealed the presence of two intrinsic molecular subtypes of urothelial carcinoma (Fig. 1B), MS1 and MS2, respectively. A significance analysis of microarrays was used to define sets of genes with higher relative expression in MS1 and in MS2 tumors, MS1_UP and MS2_UP, respectively (Supplementary Table S3). Each tumor was then assigned a gene signature score based on the average expression level of the genes in the respective gene sets. A scatter plot of these scores revealed two distinct groups of tumors, one dominated by T4 and one by ≥T2 cases whereas the T1 cases were equally distributed between the two groups (Fig. 1C). Thus, the genes that define the MS1 and MS2 subtypes clearly separate the tumors in two distinct groups.

We then analyzed the data sets of Sanchez-Carbayo (*n* = 90), Stransky (*n* = 75), and Blaveri (*n* = 75) using the same clustering algorithms and bootstrap analyses. All three data sets produced two clearly separated groups of tumors dominated by noninvasive/low-grade and muscle-invasive/high-grade tumors (Supplementary Fig. S2A and B). The robustness of the HCA results was investigated by estimating the coclustering frequency using a bootstrap approach. The resulting coclustering frequency matrix was reorganized by HCA and revealed the presence of two intrinsic molecular subtypes of urothelial carcinoma (Fig. 1B), MS1 and MS2, respectively. A significance analysis of microarrays was used to define sets of genes with higher relative expression in MS1 and in MS2 tumors, MS1_UP and MS2_UP, respectively (Supplementary Table S3). Each tumor was then assigned a gene signature score based on the average expression level of the genes in the respective gene sets. A scatter plot of these scores revealed two distinct groups of tumors, one dominated by T4 and one by ≥T2 cases whereas the T1 cases were equally distributed between the two groups (Fig. 1C). Thus, the genes that define the MS1 and MS2 subtypes clearly separate the tumors in two distinct groups.

<table>
<thead>
<tr>
<th>Table 1. Evaluation of predictor gene signatures</th>
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<tbody>
<tr>
<td>Class</td>
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<tr>
<td>SVM-based gene signature predictions§</td>
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<tr>
<td>MS</td>
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<tr>
<td>Grade</td>
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<tr>
<td>Stage</td>
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<td>DSS</td>
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<td>Progress</td>
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<td>ROC-based gene signature predictions¶</td>
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<tr>
<td>MS</td>
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<td>Grade</td>
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<td>Stage</td>
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<td>DSS</td>
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<tr>
<td>Progress</td>
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</table>

Abbreviations: MS, molecular subtype; na, not applicable.

* Gene predictors and predictions made in the present data set with cross validation.
† Gene predictors made in the Lindgren data set and predictions made in the Sanchez-Carbayo data set.
‡ Gene predictors made in the Lindgren data set and predictions made in the Stransky data set.
§ Genes in the respective gene signatures are reported in Supplementary Table S3.
¶ Genes in the respective gene signatures are reported in Supplementary Table S4. All gene signatures contained 100 genes.

The subsequent bootstrap analyses clearly indicated the presence of two robust tumor clusters in all three data sets. Both the MS1_UP and the MS2_UP gene signatures were significantly enriched in the corresponding tumor clusters in all three data sets with *P* < 0.001 in all comparisons. Hence, the MS1 and MS2 signatures identified in the present investigation clearly define two molecular subtypes intrinsic to bladder cancer.

We used GSEA to clarify possible biological differences between the MS1 and MS2 subtypes. This analysis showed significant enrichment in the MS2 tumors for six cell cycle gene sets, for two meta-profiles derived for neoplastic and undifferentiated cells, as well as for three signatures associated with poor prognosis (Supplementary Table S4). Two gene sets related to serum response and wound healing, respectively, were also enriched in MS2 tumors as well as gene sets for E2F3 activation and for genomic instability (Supplementary Fig. S6). Taken together, the functional analysis clearly shows that the MS2...
tumors are strongly associated with aggressive growth and poor prognosis.

**Prediction of pathologic and clinical entities using expression profiles.** We then used two different algorithms, one SVM-based and one ROC-based, to predict molecular subtype assignment, grade, stage, DSS, and progression to muscle-invasive growth. Using the SVM algorithm, it was possible to predict molecular subtype assignment, grade, and stage with high performance whereas survival was predicted to a lesser extent (Table 1). Similarly, high performances were obtained for stage, grade, and molecular subtype assignment when using the independent validation data sets (Table 1). Genes included in the respective SVM predictors are listed in Supplementary Table S5. The ROC-based predictors showed excellent performance for molecular subtype, grade, and stage prediction, both in the present and in the two independent data sets, whereas DSS was predicted with moderate efficiency (Table 1). The genes included in the respective ROC predictors are listed in Supplementary Table S6. Cox regression analysis was then performed for each of the ROC-based predictors with DSS, metastasis, and progression to muscle-invasive tumors as end points (Table 2). A DSS score based on the top 100 AUC genes proved to be an excellent prognostic marker for all three end points with hazard ratios ranging from 6.5 to 7.6. The DSS predictor signature also proved to be a prognostic marker for DSS and metastasis independent of tumor grade, molecular subtype, stage, and treatment with cystectomy, respectively (Table 2). Kaplan-Meier graphs were then produced for DSS and metastasis as end points (Fig. 2). The DSS signature clearly identifies high-risk and low-risk patients independently of tumor grade and treatment with cystectomy. The DSS signature also proved to have a prognostic effect (hazard ratios, 4.6; 95% confidence interval, 1.55–14; P = 0.007) among cystectomized patients in a multivariate test when correcting for node status (N0 versus N+), and pathologic stage (pT1, pT1, and pT2 versus pT3 and pT4). Genes in the DSS signature were significantly enriched for the GO category “extracellular” and included MMP11 and SPARC that influence the structure of extracellular matrix (ECM), as well as several cell proliferation genes, e.g., BIRC5 (survivin) and CDK4, and a member of the Polycomb group gene family, EZH2.

**Genomic alterations.** FGFR3 and PIK3CA mutations were seen in 73% and 34% of the MS1 cases, respectively (Fig. 3A; Supplementary Table S7) and a total of 82% of the MS1 cases

### Table 2. Cox regression analyses using the 100 gene ROC signatures

<table>
<thead>
<tr>
<th>Signature*</th>
<th>End point/subclass†</th>
<th>Hazard ratios (95% confidence interval)</th>
<th>P‡</th>
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<tbody>
<tr>
<td>MS</td>
<td>DSS</td>
<td>2.4 (1.5–3.8)</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Metastasis</td>
<td>3.3 (1.6–6.7)</td>
<td>0.0003</td>
</tr>
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<td></td>
<td>Progression§</td>
<td>2.6 (1.4–4.5)</td>
<td>0.0012</td>
</tr>
<tr>
<td>Grade</td>
<td>DSS</td>
<td>2.7 (1.6–4.8)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Metastasis</td>
<td>4.0 (1.7–9.1)</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Progression</td>
<td>2.9 (1.5–5.6)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Stage</td>
<td>DSS</td>
<td>2.8 (1.7–4.5)</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>Metastasis</td>
<td>3.3 (1.7–6.4)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Progression</td>
<td>2.8 (1.4–5.8)</td>
<td>0.0025</td>
</tr>
<tr>
<td>DSS</td>
<td>DSS</td>
<td>7.6 (3.9–15.0)</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>Metastasis</td>
<td>7.6 (3.1–18.4)</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>Progression</td>
<td>6.5 (2.2–19.3)</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>DSS/G3</td>
<td>7.8 (3.0–20.3)</td>
<td>0.0000</td>
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<td></td>
<td>DSS/MS2</td>
<td>7.3 (2.9–18.4)</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>DSS/≥T2</td>
<td>4.0 (1.4–11.7)</td>
<td>0.0073</td>
</tr>
<tr>
<td></td>
<td>DSS/cystectomy</td>
<td>6.1 (2.0–18.7)</td>
<td>0.0012</td>
</tr>
<tr>
<td></td>
<td>Metastasis/G3</td>
<td>5.5 (1.7–17.7)</td>
<td>0.0026</td>
</tr>
<tr>
<td></td>
<td>Metastasis/MS2</td>
<td>6.2 (1.9–19.7)</td>
<td>0.0012</td>
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<tr>
<td></td>
<td>Metastasis/≥T2</td>
<td>3.1 (0.8–11.1)</td>
<td>0.0816</td>
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<tr>
<td></td>
<td>Metastasis/cystectomy</td>
<td>4.4 (1.5–13.2)</td>
<td>0.0068</td>
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<tr>
<td></td>
<td>Progression≤T1,G3</td>
<td>2.0 (0.2–19.6)</td>
<td>0.5320</td>
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</table>

Abbreviation: MS, molecular subtype.

*The same signatures as in Table 1.

† When no subclass is indicated, the reported hazard ratios correspond to the whole data set (144 cases) and the given end point. When subclass is indicated, the reported hazard ratios correspond to this subclass and the given end point.

‡ P value of log rank test.

§ Progression from non–muscle-invasive growth (T1a and T1) to muscle-invasive growth.
carried an FGFR3, a PIK3CA, or both mutations. RAS, CDKN2A, and TSC1 mutations as well as CDKN2A homozygous deletions were equally distributed between the two subtypes, whereas RB1 losses, E2F3 amplifications, and TP53/MDM2 alterations (either TP53 mutations or MDM2 genomic amplifications) were highly significant for the MS2 class of tumors (Supplementary Table S7). To identify cases with a history of genomic instability, we scored the total number of focal genomic amplifications (FGA) as identified by array-CGH (Fig. 3B). The average number of FGA in the MS2 tumors differed significantly from the average FGA in the MS1 tumors (Supplementary Table S8). Furthermore, MS2 TP53/MDM2-mutated tumors showed a significantly higher number of amplifications than MS2 TP53/MDM2wt cases. The latter group did, however, still show a significantly higher number of amplifications than MS1 cases (Supplementary Table S8). This indicates the presence of three categories of urothelial carcinoma with respect to genomic complexity in which MS2 tumors with TP53/MDM2 alterations are the most complex. Even though this would suggest a link between TP53/MDM2 alterations and genomic complexity, only 1 out of 7 (14%) MS1 cases with TP53 impairment showed FGA, whereas 36 out of 38 (95%) MS2 cases with TP53/MDM2 mutation did ($P < 0.0001$, $\chi^2$ test). Conversely, only 4 out of 37 (11%) MS1 cases with no TP53/MDM2 mutations showed focal amplifications, whereas 13 of the 19 (68%) MS2 cases without TP53/MDM2 mutations did ($P < 0.0001$). This contrasting behavior between the MS1 and MS2 subtypes suggests

Figure 2. Kaplan-Meier analyses. Kaplan-Meier graphs based on the survival signature with time to death of disease as end point in (A) patients with G3 tumors ($n = 68$) and (B) in cystectomized patients ($n = 40$). With time to metastasis as end point in (C) patients with G3 tumors and (D) in cystectomized patients. Circles, completed; crosses, censored; blue, low risk; red, high risk.
that the major difference between MS1 and MS2 are signs of genomic instability among the MS2 cases rather than the presence or absence of TP53/MDM2 alterations.

**Molecular pathologic classification of bladder cancer.**

To combine molecular classification with pathologic staging, we performed supervised HCA on T<sub>a</sub>, T<sub>1</sub>, and muscle-invasive (≥T<sub>2</sub>) cases separately using the MS1/MS2 defining genes (Fig. 4). The HCA of the T<sub>a</sub> tumors produced two well-separated clusters, referred to as T<sub>a.1</sub> and T<sub>a.2</sub>. The MS1/MS2 and DSS predictor scores for the T<sub>a.2</sub> group were mixed and moderately high/intermediate, but generally higher than for T<sub>a.1</sub> cases. The T<sub>a.1</sub> and T<sub>a.2</sub> groups did not differ with respect to the frequency of FGFR3/PIK3CA mutations (P > 0.05, χ<sup>2</sup> test with Yates correction) or TP53/MDM2 alterations (P > 0.05), but differed significantly with respect to number of tumors with FGA (P < 0.020). The T<sub>1</sub> tumors also produced two subsets, T<sub>1.1</sub> and T<sub>1.2</sub>. The T<sub>1.1</sub> group showed a significantly higher frequency of FGFR3/PIK3CA mutations (P < 0.020) and a significantly lower number of cases with FGA (P < 0.001), whereas the TP53/MDM2 mutation frequency did not differ significantly (P > 0.05) between the subgroups. The T<sub>1.2</sub> tumors did, however, show significantly higher DSS scores. Hence, the T<sub>a.2</sub> and T<sub>1.2</sub> groups resemble each other by showing FGA, higher MS1/MS2, and DSS predictor scores, and consequently, represent tumor subtypes with genomic instability and an increased risk as distinguishing features. The HCA did not segregate the ≥T<sub>2</sub> cases into distinct tumor subgroups but did, however, indicate the presence of one group of tumors with a significantly higher frequency of FGFR3/PIK3CA mutations (P < 0.003). This FGFR3/PIK3CAMut ≥T<sub>2</sub> subtype did not differ with respect to TP53/MDM2 alterations or FGA but did show lower MS1/MS2 and DSS predictor scores.

**Discussion**

In the present study, we show that urothelial carcinoma is characterized by two major molecular subtypes with distinct features at the gene expression, genomic, and gene mutation levels. The two subtypes were defined by gene expression analysis using several statistical means, including bootstrap analysis. In addition, the presence of two analogous molecular subtypes, defined by the same set of genes, was also present in three independent and previously published bladder cancer data sets. From this, we conclude that urothelial carcinoma is defined by at least two intrinsic molecular subtypes, MS1 and MS2. To further validate the existence of these subtypes, we hypothesized that the statistically obtained tumor groups should also show distinct biological features. Indeed, the GSEA analysis indicated that the MS1 and MS2 subtypes differed in many important aspects. The MS2 subtype showed significant enrichment for cell cycle–related gene sets as well as for gene sets associated with cellular transformation, genomic instability, and serum response, reflecting the more aggressive behavior of the MS2 subtype. Taken together, the molecular classification of urothelial carcinoma into MS1 and MS2 subtypes based on gene expression is not only motivated from a statistical point of view but also from a biological/clinical standpoint.

**Figure 3.** Summary of gene mutation and aCGH analyses. A, occurrence of FGFR3, PIK3CA, RAS (HRAS, KRAS, or NRAS), CDKN2A, TSC1, RB1, and E2F3 alterations. TP53/MDM2 indicates a TP53 mutation or MDM2 genomic amplification. CDKN2A mutations include both gene sequence mutations and homozygous deletions. RB1 indicates deletion at the RB1 locus and E2F3 focal amplification of E2F3. Black or gray boxes, presence of the indicated mutation. B, occurrences of FGAs. Open circles, wt TP53/MDM2; filled circles, either TP53-mutated or MDM2 gene amplified cases.
The MS1 and MS2 subtypes show distinct gene mutation patterns in which *FGFR3/PIK3CA* activating mutations are frequent and highly significant for MS1 tumors, whereas *TP53/MDM2* alterations and *RB1* losses are significant for MS2 tumors. Consequently, it is motivated to claim that the MS1 tumors are, to a great extent, dependent on signals high up in the signaling pathway hierarchy and may thus be described as receptor pathway–driven. CDKN2A, TSC1, and *RAS* mutations, on the other hand, did not show tumor subtype–specific pattern. The functional analysis of the MS2 gene expression profiles and the impaired TP53 activity seen in MS2 tumors together suggested a developmental phase involving genomic instability differentiating MS2 from MS1 tumors. To investigate this possibility, we used the total number of focal amplifications as an indicator of previous genomic instability. Indeed, MS2 tumors showed a significantly higher number of FGA than MS1 tumors. Furthermore, the MS2 tumors could be divided into two groups, one with a moderate number of amplifications and no sign of TP53 impairment, and one with TP53 impairment and an excessive number of FGA. Hence, the results suggest that a major difference between MS1 and MS2 tumors is that the latter are more likely to have passed through a phase of genomic instability, augmented in the presence of TP53 impairment. This is in line with the finding that the presence of aberrant mitoses in pathologic slides of urothelial carcinoma is associated with a poor prognosis (47). The near absence of FGA in MS1 tumors with *TP53/MDM2* mutations and the significantly higher number of FGA in MS2 *TP53/MDM2wt* cases suggests that the critical event separating MS1 tumors from MS2 tumors is the impaired TP53 activity seen in MS2 tumors, not the impairment of TP53 activity per se. It has been suggested that high-grade and invasive tumors, i.e., MS2 tumors, are governed by inactivation of the TP53 pathway and that this ultimately results in tumors with highly rearranged genomes (14). Our data, however, suggest that *TP53/MDM2* mutations are not sufficient and might not be necessary for this process to occur. Hence, it may be more precise to describe MS2 tumors as genomic instability–driven rather than driven by TP53 impairment. In this scenario, the high frequency of *TP53/MDM2* alterations seen in MS2 tumors would be a consequence of a subsequent selection for TP53-impaired cells as cells refractory to apoptotic signals are more likely to survive a phase of genomic instability than cells that are not. The findings, however, do not imply that the suggested receptor and genomic instability pathways are mutually exclusive; a substantial fraction (28%) of the *FGFR3/PIK3CA*-mutated tumors also showed FGA. On the contrary, the data suggest that the acquisition of genomic instability results in progression as 64% and 79% of the *FGFR3/PIK3CA*-mutated tumors show amplified signals.

**Figure 4.** Separate clustering of Ta, T1, and ≥T2 cases. The HCA is based on genes included in the MS1_UP and MS2_UP gene signatures. MS1/MS2, molecular subclass as determined by the bootstrap analysis: blue, MS1; red, MS2. MS1/MS2 score, as described in the text: blue, low scores; gray, intermediate; red, high scores. DSS score, as described in the text: blue, low scores; gray, intermediate; red, high scores. FGFR3/PIK3CA, presence of FGFR3 or PIK3CA mutations: green, mutation; gray, wild-type for both; white, no data. TP53/MDM2, presence of TP53 mutation or MDM2 genomic amplification: red, mutation or amplification; gray, wild-type for both; white, no data. Total no. FGA, number of focal genomic amplifications: gray, no FGA, yellow, one to two FGAs, orange, three to four FGAs, red five or more FGAs.
tumors with FGA were high grade (G3) or belonged to the MS2 class of tumors, respectively, whereas the corresponding frequencies for FGFR3/PK3CA-mutated tumors with no FGA were 13% and 8%, respectively. Furthermore, even though genomic instability ultimately will result in gross genomic/transcriptomic alterations, the original growth processes may still show an imprint in the most progressed tumors, as seen by the grouping of FGFR3/PK3CA-mutated ≥T2 tumors by the ≥T2-specific HCA.

By combining molecular classification and pathologic staging, we defined two molecular classes each of Ta and T1 tumors of which the Ta.2 and T1.2 subtypes showed a strong mark of genomic instability, and high MS1/MS2 and DSS predictor scores. Hence, these analyses molecularly describe low- and high-risk subtypes of Ta and T1 tumors based on their behavior at the genome and transcriptome levels. TP53 and FGFR3 mutation status only had a moderate effect on this division. We used two independent algorithms to produce gene signatures specific for class assignments, i.e., intrinsic molecular subtype (MS1/MS2), grade (G1, G2/G3), or stage (Ta, T1/≥T2), or for specific clinical outcomes, i.e., survival and progression. Both algorithms produced excellent prediction results for class assignments that were validated in independent and previously published bladder cancer data sets. This shows that molecular grading by gene expression profiling might be a forceful method for urothelial carcinoma tumor classification. Not only are the classifications highly accurate; each tumor is assigned an unbiased value on a continuous scale that easily identifies borderline and extreme cases. Even though the DSS signature proved to have a strong prognostic value, predictions of survival were less efficient and declined when applied to the validation data. There might be several reasons for this. One obvious reason is that the most efficient and stable predictor genes were not part of the genes present in the data sets. An additional observation is that although molecular subtype (MS1/MS2), tumor grade, and stage are tumor-specific characteristics, survival and tumor progression might also be influenced by host (patient)-specific factors. Recently, allelic variants of the RR1P1 and SIP1 genes were shown to influence the risk for breast cancer progression and metastasis (48, 49). Hence, patient genotype information might be needed to optimize predictors for progression and survival in patients with bladder cancer.

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No potential conflicts of interest were disclosed.

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Combined Gene Expression and Genomic Profiling Define Two Intrinsic Molecular Subtypes of Urothelial Carcinoma and Gene Signatures for Molecular Grading and Outcome

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