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ORIGINAL REPORT

Generation of a Concise Gene Panel for Outcome Prediction in Urinary Bladder Cancer

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The Acknowledgment and Appendix are included in the full-text version of this article; they are available online at www.jco.org. They are not included in the PDF version (via Adobe® Reader®).

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ABSTRACT

Purpose
This study sought to determine if alterations in molecular pathways could supplement TNM staging to more accurately predict clinical outcome in patients with urothelial carcinoma (UC).

Patients and Methods
Expressions of 69 genes involved in known cancer pathways were quantified on bladder specimens from 58 patients with UC (stages Ta-T4) and five normal urothelium controls. All tumor transcript values beyond two standard deviations from the normal mean expression were designated as over- or underexpressed. Univariate and multivariable analyses were conducted to obtain a predictive expression signature. A published external data set was used to confirm the potential of the prognostic gene panels.

Results
In univariate analysis, six genes were significantly associated with time to recurrence, and 10 with overall survival. Recursive partitioning identified three genes as significant determinants for recurrence, and three for overall survival. Of all genes identified by either univariate or partitioning analysis, four were found to significantly predict both recurrence and survival (*JUN*, *MAP2K6*, *STAT3*, and *ICAM1*); overexpression was associated with worse outcome. Comparing the favorable (low or normal) expression of ≥ three of four versus ≤ two of four of these oncogenes showed 5-year recurrence probability of 41% versus 88%, respectively ($P < .001$), and 5-year overall survival probability of 61% versus 5%, respectively ($P < .001$). The prognostic potential of this four-gene panel was confirmed in a large independent external cohort (disease-specific survival, $P = .039$).

Conclusion
We have documented the generation of a concise, biologically relevant four-gene panel that significantly predicts recurrence and survival and may also identify potential therapeutic targets for UC.

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INTRODUCTION

Current urothelial carcinoma (UC) management primarily depends on histologic grading and pathologic staging of the tumor.[1,2] Although these provide assessment of risk, they are unable to predict outcome in an individual patient. Molecular alterations in tumors precede visually identifiable morphologic changes and are responsible for their biologic behavior,[3,4] prognosis, and response to therapy. Therefore, histopathologic staging in UC must be complemented with molecular correlates to accurately predict clinical outcome and therapeutic response.

This study was performed on the basis of growing evidence that multiple alterations in major cancer pathways are responsible for progression of UC.[5] We profiled the expression of 69 genes involved in eight crucial cancer pathways (Appendix Table A1, online only) by standardized competitive reverse transcriptase–polymerase chain reaction (StaRT-PCR; Gene Express, Toledo, OH), quantifying absolute expressions in relation to a fixed quantity of the housekeeping gene β-actin.[6] The ultimate goal is to identify a concise marker panel that can predict clinical outcome in patients with UC; this study was designed to identify genes that would comprise such a panel.

PATIENTS AND METHODS

Patient Selection

The study cohort comprised 58 patients with UC (mean age, 69.5 years) and five normal controls. Frozen

UC tissue was obtained after radical cystectomy from 49 patients at the University of Southern California (Los Angeles, CA) and nine patients at the University of California, San Francisco (San Francisco, CA), between 1991 and 2002. These included patients with invasive (T1-4) tumors and noninvasive (Ta) tumors refractory to bladder-conserving therapies. Patients with distant metastasis at time of diagnosis were excluded. TNM staging was standardized to the 2002 American Joint Committee on Cancer recommendations.[1] Controls consisted of normal urothelium from the bladder necks of patients who had undergone radical prostatectomy for localized prostatic adenocarcinoma without bladder involvement and no history of UC.

Eight patients (13.8%) received adjuvant chemotherapy and/or radiotherapy. These included patients with high-grade recurrent noninvasive (n = 1), muscle-invasive (n = 2), extravesically extending (n = 1), and nodal metastasized (n = 4) tumors. Mean follow-up was 3.04 years (range, 0.30 to 10.44 years), during which 29 patients developed recurrent disease, and 38 patients died (Appendix, online only). UC was the cause of death in 30 patients, whereas eight patients died as a result of undocumented causes. Informed consent was obtained from all patients. The study was approved by the respective institutional review boards.

StaRT-PCR and Comparison of Tumor and Normal Gene Expression Levels

After RNA extraction by conventional TRIzol method (Invitrogen, Carlsbad, CA), cDNA was prepared, and quantitative gene expression profiling was performed by StaRT-PCR (Appendix, online only).[7,8] Each gene was reported as number of mRNA molecules expressed per 10^6 β-actin molecules.

After log transformation, each transcript expression level for all patients with UC was compared with the respective mean level in normal urothelium. Any tumor transcript level greater than two standard deviations from the mean expression level in normal urothelium was labeled as overexpressed, whereas any expression level less than two standard deviations from the mean level in normal urothelium was labeled as underexpressed. Tumor transcript expression levels falling between two standard deviations above and below the mean levels in normal urothelium were labeled as normally expressed. Thus, each tumor transcript was assigned an expression value (low, normal, or high) depending on its level compared with that of normal urothelium. Once the significant genes were identified, transcript expressions were dichotomized into favorable and unfavorable categories depending on outcomes associated with respective expression values.

Data Analysis

The clinical outcomes analyzed were time to recurrence, disease-specific survival, and overall survival (Appendix, online only). Time to recurrence was preferred over disease-specific survival, because currently most patients who die as a result of UC have documentation of disease recurrence; overall survival also accounts for cases in which cause of death is unknown and in which the impact of UC treatment may contribute to death, although disease does not recur.

Table 1. Association of Patient Demographics and Clinicopathologic Parameters With Outcome

Demographic or Characteristic	No. of Patients	Recurrence				Overall Survival			
		RR of Recurrence	95% CI*	Probability ± SE† of 5-Year Recurrence	*P**	RR of Death	95% CI*	Probability ± SE† of 5-Year Survival	*P**
Study cohort	58			0.56 ± 0.08				0.38 ± 0.07	
Age, years					.48				.49
≤ 69	28	1.00	Reference	0.47 ± 0.10		1.00	Reference	0.43 ± 0.10	
≥ 70	30	1.30	0.62 to 2.70	0.63 ± 0.10		1.24	0.64 to 2.40	0.34 ± 0.09	
Sex					.28				.28
Male	49	1.00	Reference	0.51 ± 0.08		1.00	Reference	0.39 ± 0.07	
Female	9	1.63	0.66 to 4.01	0.80 ± 0.17		1.56	0.68 to 3.59	0.33 ± 0.16	
Ethnicity‡					.84				.28
White	39	1.00	Reference	0.57 ± 0.09		1.00	Reference	0.43 ± 0.08	
Other	10	1.10	0.41 to 2.95	0.52 ± 0.16		1.53	0.68 to 3.46	0.20 ± 0.13	
Tumor stage					.42				.032
Ta	10	1.00	Reference	0.56 ± 0.17		1.00	Reference	0.63 ± 0.17	
T1-2	21	0.77	0.25 to 2.36	0.52 ± 0.14		2.07	0.67 to 6.38	0.37 ± 0.11	
T3-4	27	1.34	0.49 to 3.67	0.58 ± 0.10		3.16	1.07 to 9.32	0.30 ± 0.09	
Pathologic stage					.22				.050
Noninvasive, TaN−	10	1.00	Reference	0.56 ± 0.17		1.00	Reference	0.63 ± 0.17	
Organ confined invasive, T1-2N−	17	0.87	0.28 to 2.75	0.58 ± 0.16		2.21	0.70 to 6.95	0.34 ± 0.12	
Extravesical extension, T3-4N−	11	0.53	0.13 to 2.19	0.18 ± 0.12		2.06	0.60 to 7.05	0.45 ± 0.15	
Nodal metastases, any TN+	20	1.62	0.58 to 4.53	0.71 ± 0.10		3.55	1.16 to 10.84	0.25 ± 0.10	
Lymph-node density, %§					< .001				< .001
0	38	1.00	Reference	0.48 ± 0.11		1.00	Reference	0.46 ± 0.09	
0.1-10	10	0.89	0.30 to 2.61	0.40 ± 0.15		0.99	0.38 to 2.60	0.50 ± 0.16	
> 10	10	4.19	1.76 to 10.01	1.00		4.51	1.91 to 10.64	1.00	
Tumor grade‖					.27				.050
Low	11	1.00	Reference	0.44 ± 0.17		1.00	Reference	0.59 ± 0.16	
High	47	1.79	0.62 to 5.15	0.59 ± 0.08		2.38	0.88 to 6.43	0.34 ± 0.07	

Abbreviations: RR, relative risk; SE, standard error.
*Determined on the basis of the log-rank test.
†Greenwood SE.
‡The ethnic backgrounds of nine patients were unavailable.
§Percentage of dissected lymph nodes positive for metastasis. Mean number of lymph nodes dissected: 0%, 37.5; 0.1%-10%, 49.4; and > 10%, 32.5.
‖Bergkvist grading system.

Table 2. Genes Predictive of Recurrence and Overall Survival by Univariate Analysis*

Gene	No. of Patients†	Recurrence			Overall Survival		
		RR of Recurrence	95% CI	*P*	RR of Death	95% CI	*P*
MAPK12				.091			< .001‡
Normal	17	1.00	Reference		1.00	Reference	
Low	24	0.39	0.15 to 0.97		0.23	0.10 to 0.53	
High	17	0.73	0.30 to 1.80		0.55	0.25 to 1.23	
JUN				.026‡			.001‡
Normal	40	1.00	Reference		1.00	Reference	
Low	3	0.79	0.11 to 5.82		0.49	0.07 to 3.61	
High	10	2.97	1.18 to 7.47		3.41	1.50 to 7.75	
TNFSF10				.291			.007‡
Normal	33	1.00	Reference		1.00	Reference	
Low	9	0.49	0.14 to 1.65		0.50	0.17 to 1.48	
High	14	1.36	0.58 to 3.18		2.05	0.99 to 4.25	
STAT3				.009‡			.050‡
Normal	29	1.00	Reference		1.00	Reference	
Low	11	3.31	1.24 to 8.81		1.87	0.79 to 4.40	
High	16	3.08	1.27 to 7.47		2.38	1.12 to 5.09	
CCNA2				.540			.009‡
Normal	51	1.00	Reference		1.00	Reference	
Low	2	0.55	0.07 to 4.02		0.40	0.06 to 2.83	
High	2	2.39	0.30 to 18.99		6.03	1.27 to 28.74	
ICAM1				.338			.014‡
Normal	17	1.00	Reference		1.00	Reference	
Low	19	0.78	0.29 to 2.07		0.67	0.26 to 1.70	
High	20	1.48	0.60 to 3.64		1.87	0.82 to 4.26	
BCL2L1				.204			.015‡
Normal	6	1.00	Reference		1.00	Reference	
Low	8	0.32	0.06 to 1.72		0.12	0.01 to 1.04	
High	42	1.08	0.37 to 3.13		1.28	0.48 to 3.40	
MAP2K6				.044‡			.016‡
Normal	12	1.00	Reference		1.00	Reference	
Low	22	0.42	0.15 to 1.18		0.59	0.23 to 1.53	
High	24	1.15	0.46 to 2.89		1.58	0.65 to 3.82	
IGF1				.021‡			.147
Normal	17	1.00	Reference		1.00	Reference	
Low	29	1.13	0.47 to 2.71		1.15	0.51 to 2.57	
High	4	4.95	1.15 to 21.32		2.93	0.88 to 9.77	
SOD1				.033‡			.081
Normal	9	1.00	Reference		1.00	Reference	
Low	8	2.04	0.62 to 6.76		3.03	0.89 to 10.31	
High	34	0.62	0.24 to 1.61		1.52	0.55 to 4.21	
TGIF				.449			.047‡
Normal	31	1.00	Reference		1.00	Reference	
Low	9	0.96	0.35 to 2.65		0.81	0.31 to 2.10	
High	18	1.62	0.69 to 3.82		2.04	0.98 to 4.28	
FOSL1				.337			.051§
Normal	22	1.00	Reference		1.00	Reference	
Low	26	1.69	0.75 to 3.77		1.09	0.53 to 2.26	
High	8	1.95	0.60 to 6.36		2.75	1.08 to 7.03	
BCL2				.055§			.197
Normal	24	1.00	Reference		1.00	Reference	
Low	25	0.45	0.19 to 1.07		0.61	0.29 to 1.30	
High	9	1.40	0.56 to 3.51		1.23	0.51 to 2.95	

Abbreviation: RR, relative risk.
*Log-rank test.
†Only includes patients for whom respective gene expression values were available.
‡Statistically significant ($P \leq .050$).
§Trend toward significance ($.050 < P \leq .055$).

The log-rank test[9] was used to examine how clinical parameters and gene expression values were associated with clinical outcome. In univariate analysis, the relative risk ratio and associated 95% CI were calculated on the basis of the log-rank test.[10] To adjust for multiple comparisons and control the false-positive rate, bootstrap internal validation was performed for all genes identified by univariate analysis, thereby eliminating the possibility of overfitting or biasing conclusions on the basis of a small subset.[11] One thousand bootstrap samples of 58 observations each were drawn from the original UC cohort using simple random sampling with replacement. Selected genes were retained if associated $P \leq .050$ in more than 500 simulations.[12,13] Reported P values are two sided.

Three multivariable approaches were adopted. The first approach used nonparametric classification and regression trees generated by recursive partitioning (RP) to explore gene expression variables and separate patients into prognostic subgroups on the basis of time to recurrence and overall survival (Appendix, online only).[14,15] In the second approach, stepwise forward selection was used on the basis of the Cox proportional hazards model, stratified by pathologic stage and lymph-node density. Third, Akaike information criterion (AIC) within a Cox proportional hazards model, stratified by pathologic stage, was used to demonstrate the discriminatory ability of the gene panels.[16] A smaller AIC value indicates a more desirable panel for predicting outcome.[17] Functional pathway analysis was also conducted using Dijkstra's shortest paths algorithm (Appendix, online only).[18]

External Validation

For validation purposes, multiple public repositories were searched for expression profiling data from independent external UC cohorts that encompassed all stages and provided publicly available corresponding clinical outcome information. The study by Sanchez-Carbayo et al[19] provided such a data set online that also profiled all genes investigated in our cohort. We used the same binary outcome as defined in that study: whether the patient had died as a result of UC or had no evidence of disease at last follow-up. Because true normal urothelium was not used in this study, and adjacent normal urothelium can potentially harbor genetic alterations similar to adjacent tumor tissue,[20] the expression profiles of adjacent normal urothelium were disregarded in our analysis. The final validation cohort consisted of expression profiles from primary tumors of 91 patients with UC (mean age, 67.8 years; Appendix, online only).

After log transformation, representative probe sets on U133A GeneChips (Affymetrix, Santa Clara, CA; Appendix, online only)[21] for the 11 genes predictive for overall survival from our analysis were chosen for validation in the external data set because survival was the only clinical outcome available for this cohort. Expression of any gene below or above its median expression level in the validation cohort was considered favorable or unfavorable, respectively, in accordance with the findings from our study cohort (Appendix Table A2, online only). Pearson's χ^2 test was used to examine associations with clinical outcome.

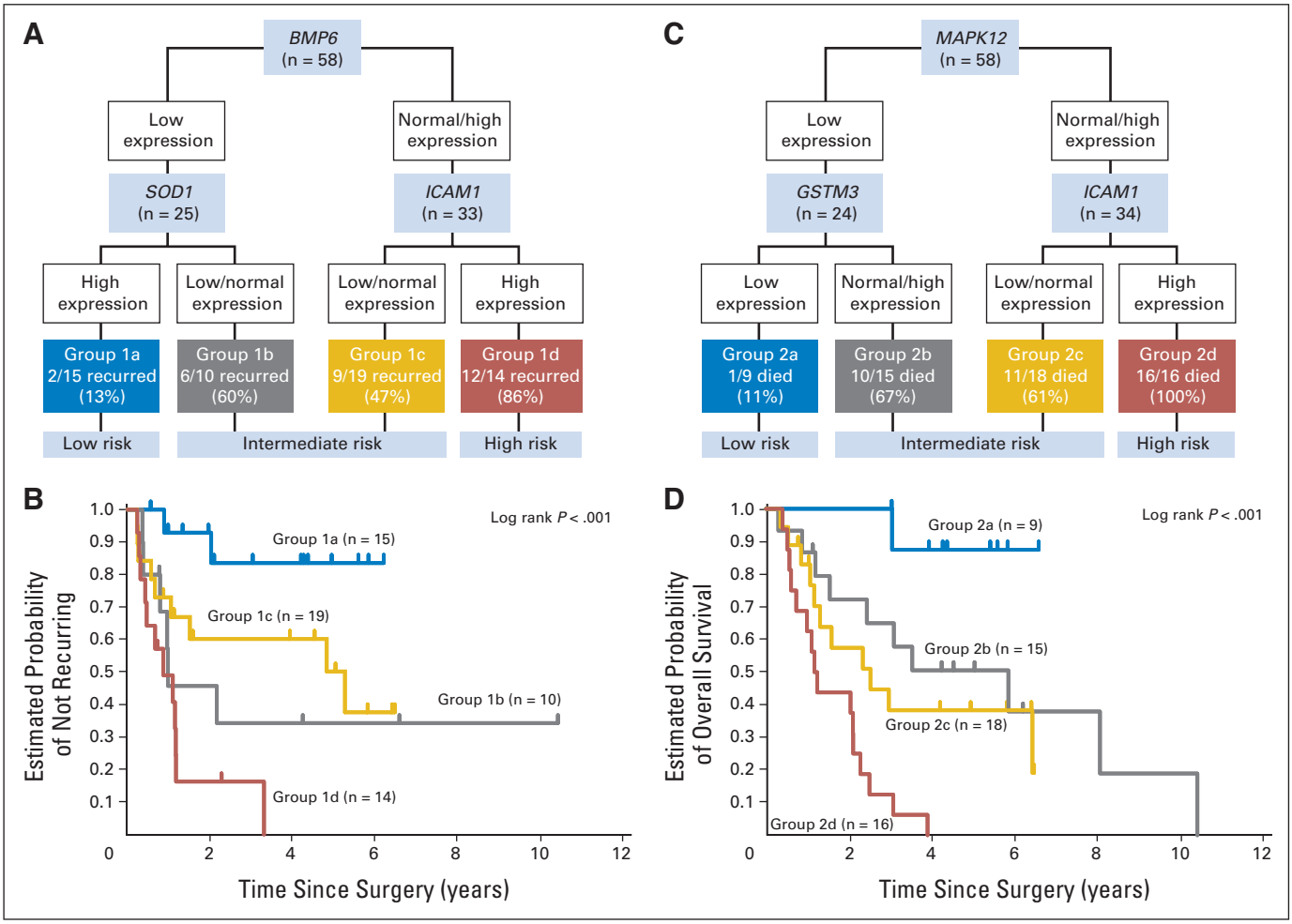

Fig 1. Recursive partitioning analysis for clinical outcome in urothelial carcinoma. (A) Expression values of *BMP6*, *SOD1*, and *ICAM1* were used to define four distinct patient groups (1a to 1d) on the basis of time to recurrence with (B) significant differences in risk of recurrence. (C) Similarly, expression values of *MAPK12*, *GSTM3*, and *ICAM1* were used to define four distinct patient groups (2a to 2d) on the basis of overall survival with (D) significant differences in survival risk.

RESULTS

Clinicopathologic Parameters and Clinical Outcome

Associations of clinicopathologic parameters of the study cohort with outcome are listed in Table 1. Pathologic stage was significantly associated with overall survival but not with time to recurrence. Interestingly, three of 10 patients with TaN− disease had postcystectomy pelvic recurrences, demonstrating an unusually aggressive clinical course. In contrast, nine of 11 patients with T3-4N− disease experienced an unusually indolent clinical course with no recurrence at last follow-up.

Individual Genes and Clinical Outcome

By univariate analysis, *STAT3* ($P = .009$), *IGF1* ($P = .021$), *JUN* ($P = .026$), *SOD1* ($P = .033$), and *MAP2K6* ($P = .044$) were significantly associated with time to recurrence (Table 2; Data Supplement, online only). *BCL2* ($P = .055$) also showed a trend toward significance for time to recurrence. The consistency of these findings was supported by bootstrap analysis that selected these transcripts in more than half of the bootstrap samples for recurrence.

MAPK12 ($P < .001$), *JUN* ($P = .001$), *TNFSF10* ($P = .007$), *CCNA2* ($P = .009$), *ICAM1* ($P = .014$), *BCL2L1* ($P = .015$), *MAP2K6* ($P = .016$), *TGIF* ($P = .047$), and *STAT3* ($P = .050$) were significantly associated with overall survival (Table 2; Data Supplement, online only). *FOSL1* ($P = .051$) also showed a trend toward significance for overall survival. Bootstrap analysis confirmed the consistency of these findings by selecting these genes in more than half of the bootstrap samples for overall survival.

Interdependent Gene Expressions and Clinical Outcome

RP analysis was performed to identify any gene that may, by itself, not be prognostically important and thus not feature in the univariate analysis but, in association with other genes, may be associated with clinical outcome. The expressions of *BMP6*, *SOD1*, and *ICAM1* were identified as joint determinants for recurrence (Fig 1A). At the end of

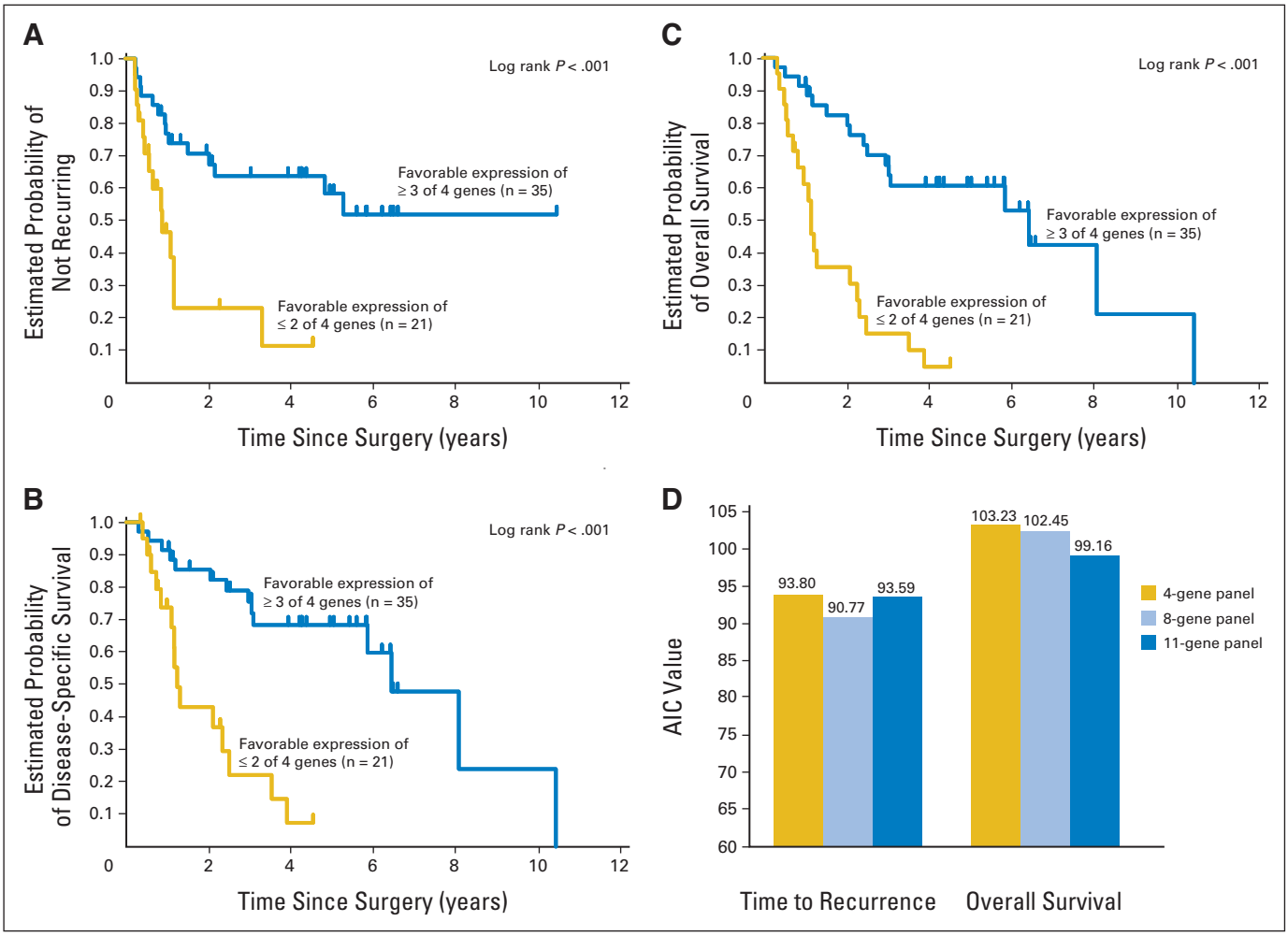

Fig 2. Generation of a predictive expression signature in urothelial carcinoma. Groups generated on the basis of low or normal (favorable) expressions of ≥ three of four or ≤ two of four genes (*JUN*, *MAP2K6*, *STAT3*, and *ICAM1*) showed significant differences in probabilities of (A) recurrence, (B) disease-specific survival, and (C) overall survival. (D) The performance of this four-gene panel compared favorably to the 8-gene (*STAT3*, *IGF1*, *JUN*, *SOD1*, *MAP2K6*, *BCL2*, *BMP6*, and *ICAM1*) and 11-gene (*MAPK12*, *JUN*, *TNFSF10*, *CCNA2*, *ICAM1*, *BCL2L1*, *MAP2K6*, *TGIF*, *STAT3*, *FOSL1*, and *GSTM3*) panels for prediction of time to recurrence and overall survival, respectively. AIC, Akaike information criterion.

the study, 87% of patients with low *BMP6* and high *SOD1* expressions (group 1a) remained recurrence free, whereas this was seen in only 14% of patients with normal or high *BMP6* and high *ICAM1* expressions (group 1d); in patients with low *BMP6* and low or normal *SOD1* expressions (group 1b) and patients with normal or high *BMP6* and low or normal *ICAM1* expressions (group 1c), intermediate recurrence rates were observed. Log-rank analysis of these four groups showed significant association with time to recurrence ($P < .001$), with group 1a demonstrating the lowest and group 1d demonstrating the highest probability of recurrence (Fig 1B).

MAPK12, *GSTM3*, and *ICAM1* were identified as joint determinants for overall survival (Fig 1C). At the end of the study, 89% of patients with low *MAPK12* and *GSTM3* expressions (group 2a) had survived, whereas 0% of patients with normal or high *MAPK12* and high *ICAM1* expressions (group 2d) had survived. Patients with low *MAPK12* and normal or high *GSTM3* expressions (group 2b) and those with normal or high *MAPK12* and low or normal *ICAM1* expressions (group 2c) had intermediate survival rates. Log-rank analysis of these four groups showed significant association with overall survival ($P < .001$), with group 2a having the best and group 2d having the worst survival probabilities (Fig 1D).

Combined Analysis of Four Common Genes

We hypothesized that the most biologically relevant genes would predict both recurrence and overall survival by univariate and/or RP analysis. *JUN*, *MAP2K6*, and *STAT3* were significantly associated with time to recurrence and overall survival by univariate analysis, and *ICAM1* was significantly associated with overall survival by univariate analysis and with recurrence and overall survival by RP analysis (Appendix Table A3, online only).

On the basis of comparison of individual gene expression patterns with outcome, low or normal expression was found to be favorable, whereas overexpression was unfavorable (Appendix Table A2, online only). This was consistent with their functions as oncogenes.[8,22,23] The study cohort was then divided into two groups: patients with favorable (low or normal) expressions of ≥ three of four genes (n = 35) and patients with favorable expressions of ≤ two of four genes (n = 21). Two patients were excluded from the analysis because they each had two favorable, one unfavorable, and one missing gene expressions and could thus not be confidently classified into either group. The 5-year recurrence probabilities in these groups were 41% and 88%, respectively ($P < .001$; Fig 2A); the 5-year disease-specific survival probabilities were 68% and 7%, respectively ($P < .001$; Fig 2B); and the 5-year overall survival probabilities were 61% and 5%, respectively ($P < .001$; Fig 2C; Table 3). To confirm that these findings were not the results of inherent differences in the pathologic stages, the analysis was repeated, stratified by each stage, and the results and patterns remained consistent (data not shown). In a sensitivity analysis, the gene panel was re-evaluated employing the Cox proportional hazards model, stratified by pathologic stage and lymph-node density. When patients with favorable expressions of ≥ three of four genes were used as the reference group, relative risks of recurrence for patients with favorable expressions of ≤ two of four genes were 3.09 and 2.63, respectively, and relative risks of death were 4.48 and 4.11, respectively. These relative risks remained statistically significant even after excluding the eight patients who received adjuvant treatment, indicating that the predictive value of these four genes was not altered by adjuvant therapy (data not shown).

Interestingly, all three patients with TaN− disease with pelvic recurrences had expression profiles consistent with high risk of recurrence (favorable expressions of ≤ two of four genes). Similarly, six of seven patients with TaN− disease and seven of nine patients with T3-4N− disease without recurrences had expression profiles consistent with low risk of recurrence (favorable expressions of ≥ three of four genes).

Relative Predictive Power of Gene Panels

To assess how much predictive power was lost on exclusion of the significant genes that were not common predictors of recurrence and

Table 3. Association of Favorable Expressions of *JUN, MAP2K6, STAT3,* and *ICAM1* With Outcome*

		Recurrence				Disease-Specific Survival				Overall Survival			
No. of Genes With Favorable Expression†	No. of Patients	RR of Recurrence	95% CI	Probability ± SE‡ of 5-Year Recurrence	*P*	RR of Death	95% CI	Probability ± SE‡ of 5-Year Survival	*P*	RR of Death	95% CI	Probability ± SE‡ of 5-Year Survival	*P*
Log-rank test					< .001				< .001				< .001
≥ 3 of 4	35	1.00	Reference	0.41 ± 0.09		1.00	Reference	0.68 ± 0.08		1.00	Reference	0.61 ± 0.09	
≤ 2 of 4	21	3.22	1.46 to 7.13	0.88 ± 0.10		4.13	1.83 to 9.32	0.07 ± 0.07		4.10	2.00 to 8.41	0.05 ± 0.05	
Cox proportional hazards model, stratified by pathologic stage					.007				.001				< .001
≥ 3 of 4	35	1.00	Reference	—		1.00	Reference	—		1.00	Reference	—	
≤ 2 of 4	21	3.09	1.37 to 6.95	—		4.11	1.75 to 9.63	—		4.48	2.09 to 9.62	—	
Cox proportional hazards model, stratified by lymph-node density					.028				.003				< .001
≥ 3 of 4	35	1.00	Reference	—		1.00	Reference	—		1.00	Reference	—	
≤ 2 of 4	21	2.63	1.11 to 6.25	—		3.92	1.59 to 9.66	—		4.11	1.89 to 8.95	—	

Abbreviations: RR, relative risk; SE, standard error.
*Two patients had two favorable, one unfavorable, and one missing gene expression values and could thus not be confidently classified into either group; they were excluded for this analysis.
†Favorable was defined as low or normal expression of the gene compared with that in normal urothelium.
‡Greenwood SE.

overall survival, AIC within a Cox proportional hazards model, stratified by pathologic stage, was used to compare the four-gene panel with the eight- and 11-gene panels, which contained genes individually predictive for recurrence and overall survival, respectively, by univariate and/or RP analysis. Expressions of these genes were dichotomized into favorable and unfavorable categories on the basis of their association with outcome (Appendix Table A2, online only), and patients with missing expression values for these genes were excluded for this analysis. Although the eight- and 11-gene panels, as expected, performed best in predicting time to recurrence and overall survival, respectively, their performance was not substantially superior to that of the four-gene panel (Fig 2D). In fact, the differences in AIC values between the four-gene panel and the best performing panels for time to recurrence and overall survival were 3.03 and 4.07, respectively. This suggests that the predictive performances of these panels were empirically comparable, because the absolute differences in AIC value were close to or less than 4.[17]

Validation of Identified Gene Panels

An independent external UC cohort,[19] profiled for gene expressions using oligonucleotide microarrays, was used to confirm the prognostic potential of the identified gene panels. Because only disease-specific survival was reported for the cohort, the 11-gene panel predictive for overall survival and the common four-gene panel were chosen for validation. Associations of clinicopathologic parameters with disease-specific survival are listed in Table 4. The cohort was divided into two groups on the basis of the 11-gene panel: patients with favorable expressions of ≥ seven of 11 genes (n = 56) and patients with favorable expressions of ≤ six of 11 genes (n = 35). Using the former as the reference group, relative risk of disease-specific death in patients with favorable expressions of ≤ six of 11 genes was 2.00 (P = .007). To assess the predictive power of the common four-gene panel, the validation cohort was again divided into two groups: patients with favorable expressions of ≥ three of four genes (n = 50) and patients with favorable expressions of ≤ two of four genes (n = 41). Using the former as the reference group, the relative risk of disease-specific death in patients with favorable expressions of ≤ two of four genes was 1.71 (P = .039).

DISCUSSION

We used a quantitative, pathway-specific approach to profile genes involved in important cellular pathways that are crucial in UC development. The choice for the final predictive panel was determined on the basis of the hypothesis that the most biologically relevant genes should be able to predict both recurrence and survival. In this study, the four-gene panel (*JUN*, *MAP2K6*, *STAT3*, and *ICAM1*) was a highly significant predictor of these outcomes, independent of standard prognostic criteria (ie, stage and lymph-node density). In addition, this panel identified high-risk patients; nearly all patients with favorable expressions of ≤ two of four genes experienced recurrence and died. The prognostic potential of this panel was additionally

Table 4. Association of Patient Demographics, Clinicopathologic Parameters, and Prognostic Gene Panels With Disease-Specific Survival in the External Validation Cohort[19]

Demographic or Characteristic	No. of Patients	Disease-Specific Survival		
		RR of Death*	95% CI	P*
Validation cohort	91			
Age, years				.54
≤ 69	52	1.00	Reference	
≥ 70	39	0.85	0.50 to 1.44	
Sex				.34
Male	63	1.00	Reference	
Female	28	0.75	0.41 to 1.38	
Tumor stage				< .001
Ta, T1-2	35	1.00	Reference	
T3-4	56	3.88	1.67 to 9.02	
Pathologic stage				< .001
Organ confined, TaN−, T1-2N−	35	1.00	Reference	
Extravesical extension, T3-4N−	31	3.39	1.39 to 8.24	
Nodal metastases, any TN+	25	4.48	1.89 to 10.62	
Tumor grade				.001
2	18	1.00	Reference	
3	73	8.63	1.27 to 58.85	
11-gene predictive panel for survival†				.007
Favorable expression of ≥ 7 of 11 genes	56	1.00	Reference	
Favorable expression of ≤ 6 of 11 genes	35	2.00	1.21 to 3.31	
4-gene predictive panel for outcome‡				.039
Favorable expression of ≥ 3 of 4 genes	50	1.00	Reference	
Favorable expression of ≤ 2 of 4 genes	41	1.71	1.02 to 2.87	

Abbreviation: RR, relative risk.
*Determined on the basis of Pearson's χ^2 test.
†*MAPK12*, *JUN*, *TNFSF10*, *CCNA2*, *ICAM1*, *BCL2L1*, *MAP2K6*, *TGIF*, *STAT3*, *FOSL1*, and *GSTM3*. Favorable defined as low expression of gene in tumor (in all duplicates, if applicable).
‡*JUN*, *MAP2K6*, *STAT3*, and *ICAM1*. Favorable defined as low expression of gene in tumor (in all duplicates, if applicable).

supported by an external data set that profiled genes using a completely different methodology, thereby demonstrating the robustness of this four-gene panel in predicting clinical outcome.

Gene expression profiles are usually generated using microarrays. These studies may involve inconsistencies in results and lack of reproducibility across platforms.[24,25] Furthermore, the output often contains more than 20 to 100 genes, which can dilute biologic and clinical relevance while increasing noise and opportunities for random chance. Although our hypothesis-driven exploration limited potential for discovery, the rational choice allowed identification of key genes and associated pathways of prognostic value.

The univariate analysis identified six genes associated with recurrence, and 10 associated with overall survival. The protein products of *IGF1*, *JUN*, *MAP2K6*, *BCL2*, *CCNA2*, *ICAM1*, *BCL2L1*, *TGIF*, and *FOSL1* have been associated with poor prognosis in several cancers, including bladder cancer.[8,22,26-31] High expressions of these genes were associated with worse prognosis, consistent with their biologic roles as oncogenes. Our study also demonstrated constitutive activation of the mitogen-activated protein kinase pathway in UC[5]; low *MAPK12* expression was associated with higher probability of overall survival. *STAT3* overexpression corresponded with poorer prognosis, consistent with observations that signal transducer and activator of transcription 3 increases the invasiveness of UC cell lines.[23] Low *SOD1* expression corresponded with decreased probability of recurrence, consistent with findings in acute myelogenous leukemia and lymphoproliferative syndromes.[32] Although tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), the protein product of *TNFSF10*, induces apoptosis, patients with increased *TNFSF10* expression had poorer overall survival. This patient subset was probably insensitive to TRAIL-mediated apoptosis, consistent with findings demonstrating that different UC cell lines have varying degrees of susceptibility to TRAIL.[33]

The RP analysis also selected *BMP6* and *ICAM1* as joint determinants of recurrence. Bone morphogenetic protein 6 promotes tumor angiogenesis, and elevated *ICAM1* expression is associated with increased metastatic potential in UC.[8,34] Patients with low *BMP6* and high *SOD1* expressions had the lowest recurrence rates, whereas those with normal or high *BMP6* and high *ICAM1* expressions had the highest. *GSTM3* was also associated with overall survival in RP analysis. *GSMT3* polymorphisms are linked to carcinogenesis, and *GSTM3* mutations are associated with increasing risk for UC.[35] In patients with low *MAPK12* expression, those with low *GSTM3* expression as well had the highest survival probability, whereas those with normal or high *GSTM3* expression had lower survival probability.

When the interrelationships between proteins transcribed from these genes were examined, nine direct and more than 150 indirect interactions were discovered (Data Supplement, online only), which highlights the importance of their crosstalk. This led us to focus on genes that could predict both recurrence and survival. Obtaining a concise prognostic marker list is crucial in such studies, because clinical applications of such panels are more cost effective and practical. Although such prognostic panels have been previously identified, they have usually featured markers from a single cellular pathway.[36,37] The four-gene panel obtained after profiling genes across multiple pathways robustly predicted clinical outcome. Additionally, the ability of this panel to accurately predict recurrence independent of stage is likely to be a useful supplement to routine staging. Furthermore, *MAP2K6* and *ICAM1* were also previously identified by our group to predict nodal metastasis in UC.[8] Validation of the four- and 11-gene panels on the external data set was consistent with AIC observations that although the 11-gene panel could expectedly better predict survival, its performance was not substantially superior to that of the four-gene panel. Moreover, the validation highlighted the robustness of the four-gene panel, independent of the platform used for profiling the genes.

In conclusion, using a multiplexed, biologically driven approach, we have identified a panel comprising *JUN*, *MAP2K6*, *STAT3*, and *ICAM1* that can predict clinical outcome in UC independent of conventional prognostic criteria and identify patients with operable UC who will experience recurrence despite undergoing definitive surgery alone. These patients would clearly benefit from additional therapy. Increasing numbers of alterations in these genes predict poorer prognosis. Additional study of this panel is warranted to better characterize its ability to identify patients at higher risk. Although limited transcripts were analyzed, this does suggest that these genes and their associated pathways may serve as promising outcome predictors and potential therapeutic targets in UC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Glossary Terms

Standardized competitive reverse transcriptase–polymerase chain reaction: Quantitative polymerase chain reaction that measures absolute expression levels of multiple genes using competitive templates of the target and reference (β-actin) genes incorporated into standardized mixtures of internal standards. Use of the same standardized mixtures potentially allows comparability of data across experiments and laboratories.

Recursive partitioning: Multivariable analysis that generates a clinically intuitive decision tree model in which the population is divided into prognostic subgroups. This is achieved through multiple dichotomous divisions on the basis of a set of independent variables.

Akaike information criterion: Measure of the goodness of fit of a statistical model that discourages overfitting and is used as a tool for model selection. For a given data set, competing models are ranked according to their Akaike information criterion value, and the one with the lowest value is considered the best. However, there is no established value above which a given model is rejected.

Dijkstra's shortest paths algorithm: Graph search algorithm that finds the path with lowest cost (ie, the shortest path) between a given node (or, in the case of functional biological networks, a given gene) and every other node.