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Combination of Molecular Alterations and Smoking Intensity Predicts Bladder Cancer Outcome: A Report from the Los Angeles Cancer Surveillance Program

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Abstract

Background—Traditional single-marker and multimarker molecular profiling approaches in bladder cancer do not account for major risk factors and their influence on clinical outcome. This study examined the prognostic value of molecular alterations across all disease stages after accounting for clinicopathological factors and smoking, the most common risk factor for bladder cancer in the developed world, in a population-based cohort.

Methods—Primary bladder tumors from 212 cancer registry patients (median follow-up, 13.2 years) were immunohistochemically profiled for Bax, caspase-3, Apaf-1, Bcl-2, p53, p21, cyclooxygenase-2, vascular endothelial growth factor, and E-cadherin alterations. “Smoking intensity” quantified the impact of duration and daily frequency of smoking.

Results—Age, pathological stage, surgical modality, and adjuvant therapy administration were significantly associated with survival. Increasing smoking intensity was independently associated with worse outcome ($P < 0.001$). Apaf-1, E-cadherin and p53 were prognostic for outcome ($P = 0.005$, 0.014 and 0.032, respectively); E-cadherin remained prognostic following multivariable analysis ($P = 0.040$). Combined alterations in all nine biomarkers were prognostic by univariable ($P < 0.001$) and multivariable ($P = 0.006$) analysis. A multivariable model that included all nine biomarkers and smoking intensity had greater accuracy in predicting prognosis than models comprising of standard clinicopathological covariates without or with smoking intensity ($P < 0.001$ and $P = 0.018$, respectively).

Conclusions—Apaf-1, E-cadherin and p53 alterations individually predicted survival in bladder cancer patients. Increasing number of biomarker alterations was significantly associated with worsening survival, although markers comprising the panel were not necessarily prognostic

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individually. Predictive value of the nine-biomarker panel with smoking intensity was significantly higher than that of routine clinicopathological parameters alone.

INTRODUCTION

Urothelial carcinoma of the bladder (UCB), the sixth most common cancer in the U.S.,¹ develops through alterations in several cellular processes.² Profiling such alterations can identify biomarkers that can improve risk assessment over traditional clinical and histopathological parameters. Early studies in UCB profiled single markers, or multiple markers within a single cellular process. More recently, we and others have used multimarker approaches to profile alterations across several pathways.^{3–6} However, most of these studies do not account for common UCB risk factors and their influence on outcome. Cigarette smoking is an established risk factor for UCB in the U.S. due to carcinogenic effects of aromatic amines in tobacco smoke.⁷

To our knowledge, this is one of the initial UCB studies to examine the prognostic value of molecular alterations and cigarette smoke exposure in a multicenter population-based cohort after accounting for routine clinicopathological criteria. Proteins central to key cellular processes commonly implicated in urothelial tumorigenesis such as apoptosis, cell-cycle regulation, inflammation, angiogenesis, and invasion were immunohistochemically profiled;⁷ several of these biomarkers have been previously identified as being individually prognostic in UCB, although none were combined in a broad multi-pathway panel.^{8–18} This was an effort to identify a marker panel that could predict outcome independent of standard criteria and smoking history.

MATERIALS AND METHODS

Patients

This Institutional Review Board-approved study reports on 212 patients with primary UCB with available archived tissues recruited through the multi-institutional Los Angeles County Cancer Surveillance Program between 1987 and 1996.¹⁹ This program is part of the California Cancer Registry and the National Cancer Institute-sponsored Surveillance, Epidemiology and End Results program that prospectively collects population-based cancer patient data. Since Los Angeles represents a microcosm of the U.S.'s shifting demographics, this population may allow better understanding of UCB nationwide. Patients with distant metastasis at diagnosis were excluded. All patients provided informed consent.

Tumor staging and grading were standardized to the American Joint Committee on Cancer and World Health Organization systems, respectively.^{20,21} 166 (78%) patients had high-grade UCB. Most non-muscle-invasive (Ta/CIS/T1, N–) tumor patients were treated by transurethral resection alone (n=99, 71%) or in combination with adjuvant therapy (n=30, 22%). In contrast, most patients with muscle-invasive (T2–4, N–) or node-positive (any T, N+) tumors were treated with radical cystectomy alone (n=35, 48%) or in combination with adjuvant therapy (n=19, 26%).

Defining cigarette smoke exposure

Patients were interviewed in person regarding smoking history up to two years before UCB diagnosis using a structured questionnaire.²² Patient was classified as a smoker if he/she smoked at least one cigarette daily for six months or longer. For smokers (n=184), duration of regular smoking until diagnosis and number of cigarettes smoked daily were also queried. To analyze impact of smoking at the pathological and molecular levels, a “smoking intensity” variable that combined raw smoking metrics was introduced, and the cohort was divided into three groups (Figure 1A): group 1 – nonsmokers or patients who smoked 20

cigarettes/day for ≤ 30 years ($n=67$, 32%); group 2 – patients who smoked for 31–40 years or >20 cigarettes/day for ≤ 30 years ($n=93$, 44%); group 3 – patients who smoked for >40 years ($n=52$, 24%). Nonsmokers were combined with light smokers in group 1 as a full sensitivity analysis revealed no substantive outcome differences between the two subgroups (log-rank $P=0.65$ between subgroups).

Immunohistochemical analysis

Serial 5 μ m sections of formalin-fixed, paraffin-embedded UCB tissues were obtained for each patient. Expressions of Bax, caspase-3, Apaf-1, Bcl-2, p53, p21, cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), and E-cadherin were analyzed by immunohistochemistry following standardized procedures (Figure 2).¹² Table 1 lists procedural specifics for each marker. Antigen retrieval was performed in all cases. Every run included established positive controls, and substitution of primary antibody with rabbit immunoglobulin fraction served as negative control. Immunoreactivity was detected by avidin-biotin complex immunoperoxidase system. Diaminobenzidine was used as chromogen, and hematoxylin was used to counterstain.

All cases were independently interpreted under light microscope by at least two investigators (APM/DH/SRS/RJC), blinded to outcome and co-readers' results. In case of discrepant results ($<5\%$ cases for each marker), the case was co-reviewed with another investigator to reach a consensus. Reasons that prevented case evaluation ($<5\%$ cases for each marker) included tissue loss or introduction of tissue folding artifacts during the staining process. Entire sections were scored for average staining intensity (absent/weak/moderate/strong), and percentage of cells showing immunoreactivity at that intensity. Markers expressions were categorized as wild-type or altered; alteration thresholds were defined through joint analysis of staining characteristics of normal urothelium, previously defined cutoffs, and biological functions of the proteins. These cutoffs were based on percentage of immunoreactive cells with/without the added value of average staining intensity, thus making the parameters semi-quantitative (Table 1).

Statistical analysis

Survival outcome was calculated from date of surgery to date of death; surviving patients were censored at last follow-up. Contingency tables and Pearson's chi-square test were used to determine associations between baseline characteristics, smoking intensity, and marker alterations. Log-rank test was used to examine univariable associations with outcome.²³ Gehan-Breslow-Wilcoxon (GBW) test was also used to examine associations between categorical variables and survival, giving more weight to events at early time points.²⁴ This was done to account for early deaths that were possibly due to disease, rather than later events that could be due to other causes. Relative risks and associated 95% CIs for univariable analyses were calculated as described by Berry et al.²⁵

Multivariable Cox proportional hazards models were constructed to assess the prognostic value of altered markers individually and in combination. Logistic regression models at the five-year survival status were constructed to assess predictive accuracies as determined by area under a receiver operating characteristic (ROC) curve when smoking and individual marker alterations were successively added to a base model that comprised standard clinicopathological predictors. Differences in predictive accuracies between two models were estimated by a z-score test statistic with the null hypothesis being that the datasets arose from binormal ROC curves with equal areas beneath them.²⁶

Reported *P* values are two-sided; level of significance was set at $P=0.050$, while $0.050 < P < 0.100$ was considered as approaching statistical significance. Analyses were performed using SAS v9.2 and Epilog Plus v1.0.

RESULTS

Clinicopathological criteria, and smoking history, individual markers and outcome

Patients included 196 (92%) Caucasians and 168 (79%) males. Median age at diagnosis was 58.9 years (range, 30.5–64.9 years). Median follow-up was 13.2 years (range, 0.5–20 years), during which 90 (42%) patients died. Associations of clinicopathological parameters with smoking intensity are shown in Table 2.

Increasing pathological stage was associated with increasing frequency of p53 ($P < 0.001$), E-cadherin ($P = 0.002$), p21 ($P = 0.022$) and Apaf-1 ($P = 0.047$) alterations. Expectedly, p53 ($P < 0.001$), E-cadherin ($P = 0.001$), and p21 ($P = 0.046$) expressions were also associated with surgery; surgical modality was associated with pathological stage ($P < 0.001$), where 129 (93%) patients with non-muscle-invasive tumors had transurethral resection, and 54 (74%) patients with muscle-invasive or nodal metastasized disease had radical cystectomy.

Age ($P < 0.001$), pathological stage ($P < 0.001$), surgical modality ($P = 0.050$) and adjuvant therapy administration ($P < 0.001$) were associated with survival by log-rank analysis. These significant associations were also confirmed at early time points by the GBW test (Table 3).

Associations of smoking with individual markers and outcome

Patients who smoked >30 years had greater risk of COX-2 alterations ($P = 0.020$; data not shown). Number of cigarettes smoked daily was associated with Bax expression ($P = 0.008$; data not shown). Increased smoking intensity was associated with poor outcome (log-rank, GBW $P < 0.001$; Table 3, Figure 1B). Additionally, increase in smoking duration and number of cigarettes smoked daily were associated with worse prognosis (log-rank < 0.001 and $P = 0.003$, respectively). Smoking intensity was not associated with individual marker alterations.

Individual marker alterations

To investigate whether individual protein alterations could predict downstream effects, associations of perturbations in various molecular pathways with each other were examined. Expression patterns of several markers were associated with each other within the same and across different cellular processes (data not shown). Bax, p21, and p53 alterations were associated with concomitant alterations in caspase-3 ($P < 0.001$), Apaf-1 ($P = 0.028$) and E-cadherin ($P = 0.002$), respectively. However, Bcl-2 alterations were associated with wild-type caspase-3 ($P < 0.001$) and Bax ($P = 0.032$) phenotypes, thereby suggesting possible exclusivity of anti-apoptotic and pro-apoptotic processes in these tumors. These expected associations of alterations across various pathways indicated that our observations were biologically valid.

Individual markers and clinical outcome

Apaf-1 ($P = 0.005$), E-cadherin ($P = 0.014$) and p53 ($P = 0.032$) alterations were associated with poor prognosis by log-rank analysis; these associations were also significant by the GBW test (Table 3). p21 ($P = 0.063$) and Bax ($P = 0.067$) expressions also approached significance by log-rank analysis; the prognostic value of p21 was significant at early time points (GBW $P = 0.044$).

A multivariable Cox proportional hazards model was then created to include all baseline clinicopathological prognostic criteria, smoking intensity, and individual marker alterations (Table 3). Pathological stage ($P=0.003$) and smoking intensity ($P<0.001$) were the only baseline characteristics that still retained their associations with outcome. E-cadherin was the only marker that remained predictive for outcome following this analysis ($P=0.040$). Caspase-3 ($P=0.058$), Apaf-1 ($P=0.067$) and Bax ($P=0.068$) alterations also approached significance for outcome by multivariable analysis.

Prognostic performance of combined molecular alterations relative to baseline parameters

We next proceeded to analyze the combined prognostic value of alterations in all nine biomarkers, irrespective of their individual prognostic potential. Patients were assigned to one of three groups: 3 markers altered ($n=52$), 4–5 markers altered ($n=129$), and 6–9 markers altered ($n=31$). When thus divided, increasing number of marker alterations was associated with poor prognosis (log-rank, test for trend, GBW $P<0.001$; Table 3, Figure 3A). When subset analyses were performed using this categorization, increasing number of marker alterations were also associated with poor outcome in patients with non-muscle-invasive (log-rank $=0.022$, GBW $P=0.012$) and muscle-invasive (log-rank $P=0.001$, GBW $P=0.002$) UCB. The association between number of altered markers and outcome remained significant in a multivariable Cox proportional hazards model that included all baseline clinicopathological covariates and smoking intensity ($P=0.006$; Table 3).

Finally, to determine if there was any substantially increased prognostic value to adding smoking intensity and alterations in the nine biomarkers over standard clinicopathological covariates, a base logistic regression model was created using age, pathological stage, surgical modality and adjuvant therapy administration (Figure 3B). The accuracy of this model alone in predicting survival, as measured by area under the ROC curve, was 75.6%. When smoking intensity was added to this model, its predictive accuracy increased to 80.9%. This increase in predictive accuracy, based solely on the addition of smoking intensity, was statistically significant ($P=0.012$, Figure 3C). Individual marker alterations were then added successively to the model, beginning with E-cadherin and progressively adding one marker at a time based on their decreasing significance of association with outcome as determined by the multivariable Cox analysis that included individual markers (Table 3). With each new marker addition, the predictive accuracy of each successive model continued to increase significantly over the base model, irrespective of the marker's individual association with outcome. Predictive accuracy of the final model that included base model covariates, smoking intensity and alterations in all nine markers was 85.4%, which was significantly higher than the base model alone ($P<0.001$; Figure 3B,C). Moreover, the final model's predictive accuracy was significantly higher than the model consisting of baseline covariates and smoking intensity ($P=0.018$).

DISCUSSION

This study employed a multi-pathway-based approach to profile alterations in nine proteins involved in cell-cycle regulation, apoptosis, inflammation, angiogenesis and invasion – crucial processes in UCB pathogenesis.⁷ Associations of these alterations with smoking history and outcome were examined across all disease stages in a population-based cohort. The study highlighted the prognostic importance of smoking intensity, a novel variable that accounted for incrementally harmful effects of smoking. It also identified Apaf-1, E-cadherin and p53 as individual markers of UCB outcome, with E-cadherin remaining prognostic after multivariable modeling. Importantly, combination of alterations in all nine markers was robustly prognostic even after multivariable analysis. As with our previous observations,^{5,13} this study indicates that number of alterations is directly proportional to UCB outcome in a progressive fashion, confirming that accumulation of molecular

aberrations is more important than the individually measured alterations themselves. Patients with alterations in 6–9 markers had a very poor outcome, and these individuals clearly need more aggressive management. However, unlike prior investigations,^{5,13} this study also indicates that the nine-biomarker panel can be predictive of outcome even when markers in the panel may not be individually prognostic.

Tobacco smoking is the most important risk factor for UCB in the developed world.⁷ While traditional parameters such as smoking status and duration, and number of cigarettes smoked daily are reasonable measures of smoking habit, they may not gauge the severity and chronicity of exposure in a fashion most relevant to bladder tumorigenesis. Measurement of “pack years”, which is based on number of years smoked and average packs smoked daily, does not distinguish between individuals who smoke fewer packs for a longer duration from those who smoke more packs for a shorter duration, thereby poorly assessing chronicity of exposure.²⁷ To reconcile this issue, we defined “smoking intensity” risk strata based on traditional smoking history parameters; patients were categorized into groups organized in the order of increasing detrimental effects of tobacco smoke. While association of smoking and UCB incidence is established,²² our analysis also revealed that increasing smoking intensity influences the disease’s biological behavior with adverse effects on prognosis.

Previous studies have examined proteins interrogated in this report, either singly or in combination, with respect to UCB prognosis.^{8–18} However, none of these studies accounted for the influence of smoking on protein alterations or outcomes in their cohorts. Furthermore, these studies did not profile such a comprehensive biomarker panel involving multiple cellular processes on a single cohort. Survival analysis after adjustment of clinicopathological and smoking parameters permits characterization of biomarker panels that predict outcome independent of these prognostic criteria, thereby allowing individualized patient management.

Associations between individual markers revealed interactions between apoptotic, cell-cycle regulation and invasion processes in urothelial tumorigenesis. Besides validating our multi-pathway-based approach towards candidate biomarker selection, this also served as a biological assessment of data quality in this study. p53 alterations were absent in low-grade Ta tumors, and increased to 41% of all muscle-invasive tumors, confirming the protein’s role in more aggressive disease (data not shown).^{28,29}

E-cadherin, p53 and Apaf-1 were individual outcome predictors. Decreased E-cadherin expression has been associated with recurrence, progression and poor survival in UCB.¹⁸ Previous studies have suggested a role for p53 in predicting prognosis and chemotherapeutic response, although this observation was not validated in a prospective trial.^{11,30,31} While prior reports have not described a prognostic role of Apaf-1 in UCB, studies on this pro-apoptotic protein were limited in terms of cohort size and range of pathological stages examined.¹⁰

Multivariable analysis found E-cadherin alterations prognostic independent of baseline clinicopathological parameters and smoking. Sequential addition of individual marker alterations revealed that a composite model containing all nine biomarkers could more significantly and accurately predict UCB outcome than baseline parameters without or with smoking intensity. This underscores the added and independent prognostic value of these biomarkers over routine clinicopathological parameters. The prognostic importance of this nine-biomarker panel was apparent as increasing number of alterations was associated with worsening survival, irrespective of which specific proteins harbored those alterations.

This study is unique in examining molecular alterations in primary UCB associated with smoking in a population-based cohort where long-term survival was assessed. Protein

expression was examined on serial tumor sections rather than tissue microarrays, which allowed examination of areas of heterogeneity that could have been potentially missed on microarray cores. This allowed semi-quantitative marker assessment using percentage of cells showing altered protein expression and relative preponderance of altered molecules measured as staining intensity in an average tumor cell.

Variables that could potentially influence survival, including age, pathological stage, smoking, and treatment type, were accounted for by multivariable analysis. While we were limited by the availability of disease-specific mortality, the long follow-up ensured that all events due to disease were included in the death statistics. Furthermore, the GBW test was equally or more significant in most cases where log-rank analysis was significant for outcome, indicating that the biomarkers were truly and specifically associated with UCB prognosis rather than merely related to simple measures of longevity.

In conclusion, these findings can potentially impact UCB management across all stages. Documentation of tobacco-use history is important; increasing smoking intensity was identified as a unique independent variable that may be associated with poor prognosis. Furthermore, the nine-biomarker panel can be employed as a tool to identify patients in need of more aggressive treatment, independent of routine clinicopathological parameters or smoking history. While validation of this panel is needed, this study clearly demonstrates that a multi-pathway-based approach to constructing rational marker panels holds potential for future UCB management.

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Figure 1. Impact of smoking intensity on bladder cancer prognosis

(A) A composite smoking intensity variable was defined using smoker status, duration of smoking and number of cigarettes smoked daily. The upper solitary rhomboid represents nonsmokers. The lower 3×3 rhomboid grid represents smokers, subdivided based on duration of smoking in years along one diagonal axis and number of cigarettes smoked per day along the other diagonal axis. Based on worsening exposure to cigarette smoke, patients in each rhomboid were categorized into **Group 1** (nonsmokers or patients who smoked ≤20 cigarettes/day for ≤10 years, n=67; **green**), **Group 2** (patients who smoked for 31–40 years or >20 cigarettes/day for ≤10 years, n=93; **blue**), and **Group 3** (patients who smoked for >40 years, n=52; **red**). (B) Patients with increased smoking intensity, represented by a higher group number, had significantly poorer clinical outcomes.

Figure 2. Representative immunoreactivity photomicrographs for interrogated proteins Serial 5 μm sections of formalin-fixed paraffin-embedded primary tumor tissues were assayed for (A) Bax, (B) caspase-3, (C) Apaf-1, (D) Bcl-2, (E) p53, (F) p21, (G) COX-2, (H) VEGF, and (I) E-cadherin. All photomicrographs are at 200 $\times$ magnification. **Black scale bar** represents 50 μm .

Figure 3. Combined prognostic value of the nine-biomarker panel in bladder cancer

(A) Combined analysis of the entire cohort showed that increasing number of molecular alterations was significantly associated with worse prognosis. (B) Predictive accuracies (on Y axis) were calculated when smoking intensity and individual marker alterations were sequentially added in order of decreasing multivariable significance of association with outcome to a base model of standard predictors including age, pathological stage, surgery and adjuvant therapy administration (on X axis). (C) When measured as area under receiver operating characteristic curve, the predictive accuracy of the final model that included all nine biomarker alterations, smoking intensity and baseline clinicopathological characteristics (green) was significantly higher at 85.4%, compared to 75.6% for the base model alone (red) and 80.9% for a model that included baseline clinicopathological metrics and smoking intensity (blue).

Table 1

Antibodies used and immunohistochemical interpretations.

Marker	Cellular process	Clone	Antibody dilution	Definition of altered status	Cases evaluated (%)
Bax ^{1,a}	apoptosis	2D2 [*]	1:400	Absent/weak staining, or 30% cells stained moderately, or <10% cells stained strongly	212 (100)
Caspase-3 ^{1,a}	apoptosis	3CSP03 [*]	1:200	Absent/weak staining, or 30% cells stained moderately, or <10% cells stained strongly	212 (100)
Apaf-1 ^{1,a}	apoptosis	_8	1:200	75% cells stained	212 (100)
Bcl-2 ^{2,a}	apoptosis	124 [*]	1:40	>25% cells stained weakly, or 10% cells stained moderately, or any strong staining	211 (99)
p53 ^{3,b}	cell-cycle regulation	1801 [*]	1:3000	>25% cells stained strongly	206 (97)
p21 ^{3,b}	cell-cycle regulation	EA10 [*]	1:100	<10% cells stained	211 (99)
COX-2 ^{1,a}	inflammation	SP21 [¶]	1:100	>75% cells stained weakly, or >50% cells stained moderately, or >25% cells stained strongly	211 (99)
VEGF ^{1,a}	angiogenesis	JH121 [*]	1:100	>75% cells stained	203 (96)
E-cadherin ^{4,c}	invasion	4A2C7 [*]	1:100	Absent staining, or 25% cells stained weakly, or <10% cells stained moderately	211 (99)

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^{*} Mouse; monoclonal

[§] Rabbit; polyclonal

[¶] Rabbit; monoclonal

^a Antigen localized to cytoplasm.

^b Antigen localized to nucleus.

^c Antigen localized to cell membrane.

Table 2

Associations between demographic factors and clinicopathological parameters, and smoking intensity.

	n	Smoking intensity [¶]			P*
		Group 1 n (row %)	Group 2 n (row %)	Group 3 n (row %)	
All patients	212	67 (32)	93 (44)	52 (24)	
Age					<0.001
<60 years	121	48 (40)	62 (51)	11 (9)	
60 years	91	19 (21)	31 (34)	41 (45)	
Pathological stage					0.20
Non-muscle-invasive	139	47 (34)	64 (46)	28 (20)	
Muscle-invasive	55	13 (24)	24 (43)	18 (33)	
Nodal metastasis	18	7 (39)	5 (28)	6 (33)	
Surgery					0.38
Transurethral resection	148	51 (34)	63 (43)	34 (23)	
Radical cystectomy	64	16 (25)	30 (47)	18 (28)	
Therapeutic intervention					0.31
Surgery only	150	52 (35)	62 (41)	36 (24)	
Surgery+Adjuvant therapy	62	15 (24)	31 (50)	16 (26)	

[¶] Group 1, nonsmokers or patients who smoked < 20 cigarettes/day for < 30 years; Group 2, patients who smoked for 31–40 years or >20 cigarettes/day for > 30 years; Group 3, patients who smoked for >40 years.

* Pearson's chi-square test.

Table 3
Associations between demographic factors, clinicopathological parameters and marker alterations, and clinical outcome.

	n	Clinical outcome (univariable)			Clinical outcome (multivariable ¹)			Clinical outcome (multivariable ²)		
		Relative risk of dying (95% CI)	P [§]	P [†]	Relative risk of dying (95% CI)	P [*]	Relative risk of dying (95% CI)	P [*]	Relative risk of dying (95% CI)	P [*]
Age										
<60 years	121	1.00	<0.001	<0.001		0.21		0.29		
60 years	91	2.11 (1.39, 3.20)			1.00		1.29 (0.81, 2.05)		1.00	
Pathological stage										
Non-muscle-invasive	139	1.00	<0.001	<0.001	1.00	0.003	1.00	0.010	1.00	
Muscle-invasive	55	1.56 (0.98, 2.49)			1.44 (0.71, 2.91)		1.49 (0.78, 2.85)			
Nodal metastasis	18	4.36 (2.32, 8.18)			5.83 (2.06, 16.54)		4.53 (1.72, 11.94)			
Surgery										
Transurethral resection	148	1.00	0.050	0.012	1.00	0.43	1.00	0.34	1.00	
Radical cystectomy	64	1.52 (0.99, 2.34)			0.76 (0.38, 1.51)		0.73 (0.38, 1.40)			
Therapeutic intervention										
Surgery only	150	1.00	<0.001	<0.001	1.00	0.08	1.00	0.024	1.00	
Surgery+Adjuvant therapy	62	2.06 (1.35, 3.13)			1.53 (0.96, 2.46)		1.69 (1.08, 2.66)			
Smoking intensity[¶]										
Group 1	67	1.00	<0.001	<0.001	1.00	<0.001	1.00	<0.001	1.00	
Group 2	93	2.15 (1.17, 3.93)			2.59 (1.29, 5.21)		2.18 (1.15, 4.13)			
Group 3	52	5.76 (3.08, 10.76)			6.11 (3.02, 12.37)		5.80 (2.87, 11.71)			
Bax										
Wild-type	33	1.00	0.067	0.07	1.00	0.068	–	–	–	
Altered	179	1.88 (0.94, 3.74)			1.92 (0.91, 4.06)		–	–	–	
Caspase-3										
Wild-type	38	1.00	0.30	0.48	1.00	0.058	–	–	–	
Altered	174	0.77 (0.46, 1.28)			0.56 (0.32, 1.00)		–	–	–	
Apaf-1										
Wild-type	91	1.00	0.005	0.002	1.00	0.067	–	–	–	
Altered	121	1.82 (1.18, 2.81)			1.57 (0.96, 2.57)		–	–	–	
Bcl-2										
			0.36	0.35		0.20	–	–	–	

	n	Clinical outcome (univariable)			Clinical outcome (multivariable ¹)			Clinical outcome (multivariable ²)		
		Relative risk of dying (95% CI)	P [§]	P [†]	Relative risk of dying (95% CI)	P [*]	Relative risk of dying (95% CI)	Relative risk of dying (95% CI)	P [*]	P [*]
Wild-type	159	1.00			1.00			–		
Altered	52	1.24 (0.77, 2.00)			1.41 (0.84, 2.36)			–		
p53			0.032	0.014		0.68				
Wild-type	161	1.00			1.00			–		
Altered	45	1.66 (1.04, 2.66)			1.12 (0.66, 1.90)			–		
p21			0.063	0.044		0.77				
Wild-type	188	1.00			1.00			–		
Altered	23	1.73 (0.95, 3.14)			1.12 (0.53, 2.34)			–		
COX-2			0.40	0.45		0.23				
Wild-type	66	1.00			1.00			–		
Altered	145	1.22 (0.77, 1.94)			1.38 (0.81, 2.37)			–		
VEGF			0.13	0.10		0.85				
Wild-type	50	1.00			1.00			–		
Altered	153	1.49 (0.88, 2.50)			1.05 (0.61, 1.81)			–		
E-cadherin			0.014	0.009		0.040				
Wild-type	196	1.00			1.00			–		
Altered	15	2.23 (1.15, 4.32)			2.34 (1.09, 5.02)			–		
Number of altered markers			<0.001	<0.001		–			0.006	
3	52	1.00			–			1.00		
4–5	129	1.82 (1.03, 3.21)			–			1.38 (0.77, 2.50)		
6–9	31	4.97 (2.54, 9.72)			–			3.22 (1.52, 6.84)		

¹Model includes demographic/clinicopathological parameters, and individual marker alterations.

²Model includes demographic/clinicopathological parameters, and number of altered markers.

[§]Log-rank test.

[†]Gehan-Breslow-Wilcoxon test.

^{*}Cox proportional hazards model.

^{††}Group 1, nonsmokers or patients who smoked <20 cigarettes/day for <30 years; Group 2, patients who smoked for 31–40 years or >20 cigarettes/day for >30 years; Group 3, patients who smoked for >40 years.

CI: confidence interval.