Promoter Hypermethylation Identifies Progression Risk in Bladder Cancer

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Abstract **Purpose:** New methods to accurately predict an individual tumor behavior are urgently required to improve the treatment of cancer. We previously found that promoter hypermethylation can be an accurate predictor of bladder cancer progression, but it is not cancer specific. Here, we investigate a panel of methylated loci in a prospectively collected cohort of bladder tumors to determine whether hypermethylation has a useful role in the management of patients with bladder cancer.

Experimental Design: Quantitative methylation-specific PCR was done at 17 gene promoters, suspected to be associated with tumor progression, in 96 malignant and 30 normal urothelial samples. Statistical analysis and artificial intelligence techniques were used to interrogate the results.

Results: Using log-rank analysis, five loci were associated with progression to more advanced disease (*RASSF1a, E-cadherin, TNFSR25, EDNRB*, and *APC*; $P < 0.05$). Multivariate analysis revealed that the overall degree of methylation was more significantly associated with subsequent progression and death (Cox, $P = 0.002$) than tumor stage (Cox, $P = 0.008$). Neuro-fuzzy modeling confirmed that these five loci were those most associated with tumor progression. Epigenetic predictive models developed using artificial intelligence techniques identified the presence and timing of tumor progression with 97% specificity and 75% sensitivity.

Conclusion: Promoter hypermethylation seems a reliable predictor of tumor progression in bladder cancer. It is associated with aggressive tumors and could be used to identify patients with either superficial disease requiring radical treatment or a low progression risk suitable for less intensive surveillance. Multicenter studies are warranted to validate this marker.

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New prognostic methods are required to improve the care of patients with cancer. Current pathologic and radiological analyses of tumors often lead to the under or overstaging of disease extent. Consequently, there are delays in the initiation of radical treatment for aggressive disease and overtreatment for patients who could be managed conservatively. In bladder urothelial cancer, delays to radical treatment occur for many reasons, including a failure of endoscopic resection to detect muscle involvement in up to 50% of invasive tumors (1), resulting in treatment failure and mortality from systemic disease (2). As most tumors are initially superficial, it is likely that recognition of disease potential and prompt treatment at this early stage would significantly improve survival rates (2). With regard to overtreatment, the majority of patients with superficial urothelial cancer have tumors that will not progress to muscle invasion and are not life threatening. However, the current criteria for identifying patients at high risk of tumor progression are not sufficiently robust to allow the cessation of surveillance of those with low-risk disease. Consequently, all patients with superficial bladder cancer undergo regular check cystoscopy, with discomfort, occasional morbidity, and significant cost. Bladder cancer is indeed one of the most expensive human malignancies to manage (3).

Improvements in the prediction of tumor behavior could be made with the use of novel molecular biomarkers. To date, the clinical effect of these has been limited due to the lack of sufficiently robust molecules and methodologic difficulties. Studies have shown only limited additional prognostic benefit for individual biomarkers such as p53 (4), suggesting that a biomarker panel will be required to improve upon the current accuracy of clinicopathologic variables. This panel could consist of representative genes from key molecular pathways or a selection reflecting the underlying molecular state of a cancer.

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Table 1. Details of the tumors studied in this report and their progression status

	n (%)	Follow up*	Progression, *n* (%)	
			By grade	By stage
Grade				
1	20 (21)	27	2 (10)	3 (15)
2	33 (34)	31	0 (0)	11 (33)
3	38 (40)	22	0 (0)	17 (45)
Stage				
pT_{is}	5 (5)	30	0 (0)	0 (0)
pT_a	52 (54)	30	2 (4)	14 (27)
pT_1	18 (19)	26	0 (0)	5 (28)
pT_{2-4}	21 (22)	18	0 (0)	12 (57)

*Mean period of surveillance following tumor resection (in months).

Recently, it has become apparent that many molecular events in cancer are at the epigenetic level, including gene promoter hypermethylation. Several groups have found this to occur commonly in cancer and found that the presence of a methylator phenotype (CIMP; refs. 5, 6) to be associated with an adverse clinical outcome (7–11). Promoter methylation seems an attractive biomarker as it occurs mostly in aggressive tumors and can be identified at an early stage of the disease (12–14). For example, in superficial urothelial cancer, methylation is significantly associated with progression to muscle invasion and death (7, 14). Methylation also has practical benefits over other molecular biomarkers. First, as changes in methylation occur globally within a cancer DNA, a few selected loci could reflect the entire epigenome status. Second, hypermethylation always occurs in the same genomic region, making targeting easy. Third, hypermethylation can be determined from DNA, avoiding the problems of using RNA or immunohistochemistry. Finally, the abnormal result is a positive signal, reducing the problem of normal cell contamination. However, methylation is not cancer specific as it is known to increase with age at some loci (5, 6) and can be detected in biological fluids from patients without malignancy (15, 16).

To determine whether promoter methylation has a useful role in managing urothelial cancer, we evaluated a panel of genes previously found or suspected to be associated with adverse clinical outcome in a prospectively collected urothelial cancer cohort. The results and their implication are discussed.

Materials and Methods

Patients and tumors. Ninety-six patients with new bladder urothelial cancer were studied in this report. Each had their tumor excised by either trans-urethral resection or radical cystectomy at the Royal Hallamshire Hospital, Sheffield between July 1999 and January 2005. Representative tumor samples were collected at the time of surgery and immediately stored in liquid nitrogen until use. Normal-looking bladder urothelium was also collected from 23 of these patients and from seven others with urothelial cancer (who had inadequate tumor tissue for this study). Frozen sections were obtained and examined to confirm the diagnosis of each sample. The patients were typical of the urothelial cancer population with 66 (69%) males, 73 (76%) ex or current smokers, and a median age of 77 years (range, 48-92 years). The collected tumors were representative of the urothelial cancer tumor spectrum (Table 1). Following resection, all patients underwent relapse surveillance (median patient follow-up was 24 months) with frequency and method stratified according to disease potential (17). Forty-two (44%) patients had tumor recurrence (a new tumor with a better or similar stage or grade to the initial); 32 (33%) patients had progression [to a worse stage (n = 30) or grade (n = 2)]; and 21 (22%) patients died from urothelial cancer. South Sheffield local ethics committee approval was obtained before the commencement of this study, and all samples were collected after obtaining informed consent.

Hypermethylation analysis. Tumor and normal DNA were extracted from 30 μm of tissue using phenol chloroform extraction following thawing, microdissection, and Proteinase K digestion. For methylation analysis, the 1 to 2 μg DNA was treated with sodium bisulfite using the CpGenome kit (Chemicon, Hampshire, United Kingdom) according to manufacturer's instructions. Quantitative methylation-specific PCR was used to determine the methylation status of the promoter region of 17 candidate genes and β-actin. The β-actin primers were specific for bisulfite treatment but not methylation. Only samples with a positive β-actin result were analyzed for promoter methylation. Unmethylated PCR primers were not used as we have previously found β-actin quantification to be equivalent to them, reliable for analysis, and applicable to each locus (data not shown; ref. 14). Quantitative methylation-specific PCR was done using the ABI prism 7900 detection system. A threshold of $0.2\Delta R_n$ was used to determine the presence of a PCR product. The primers, fluorescence-labeled probes, and PCR conditions are described in detail elsewhere (14–16). Seventeen gene promoters were analyzed in this study (Table 2; Supplementary Data). These were chosen as they have previously been found or were suspected to predict tumor progression (7, 11, 14, 15, 18–25).

Statistical and artificial intelligence analyses. The presence and relative quantity of methylation were measured for each sample at each promoter. The quantity was expressed as a ratio, based on the level of fluorescence released by the quantitative methylation-specific PCR reaction, compared with the β-actin fluorescence level of that sample (16). In addition, a methylation index [(number of loci methylated / total number of loci analyzed) × 100] was calculated for each sample. For analysis, two-tailed statistical studies were done using SPSS, version

Table 2. Methylation frequencies at the 17 gene promoters in normal and malignant urothelium

	Urothelial tumor, *n* (%)	Normal urothelium, *n* (%)	χ^2, *P*
RASSF1A	52 (54.2)	14 (46.7)	
E-cadherin	39 (40.6)	6 (20.0)	0.04*
WIF-1	54 (56.3)	4 (13.3)	0.0001
TNFRSF25	72 (75.0)	5 (16.7)	0.0001
EDNRB	64 (66.7)	3 (10.0)	0.0001
CDH4	14 (14.6)	0 (0.0)	0.027*
APC	30 (31.3)	1 (3.3)	0.002
P16	1 (1.0)	0 (0.0)	0.02*
DAPK	21 (21.9)	1 (3.3)	
RARβ	2 (2.1)	0 (0.0)	
CYCLIND2	21 (21.9)	4 (13.3)	
TIMP3	22 (22.9)	3 (10.0)	
MGMT	36 (37.5)	0 (0.0)	0.0001
BCL2	43 (44.8)	1 (3.3)	0.0001
hTERT	20 (20.8)	0 (0.0)	0.006
IGFBP3	41 (42.7)	4 (13.3)	0.002
HIC-1	21 (21.9)	5 (16.7)	

NOTE: In 8 of 17 loci, higher rates of methylation were present in malignant than in normal urothelial tissue.
*Not significant after Bonferonni's correction.

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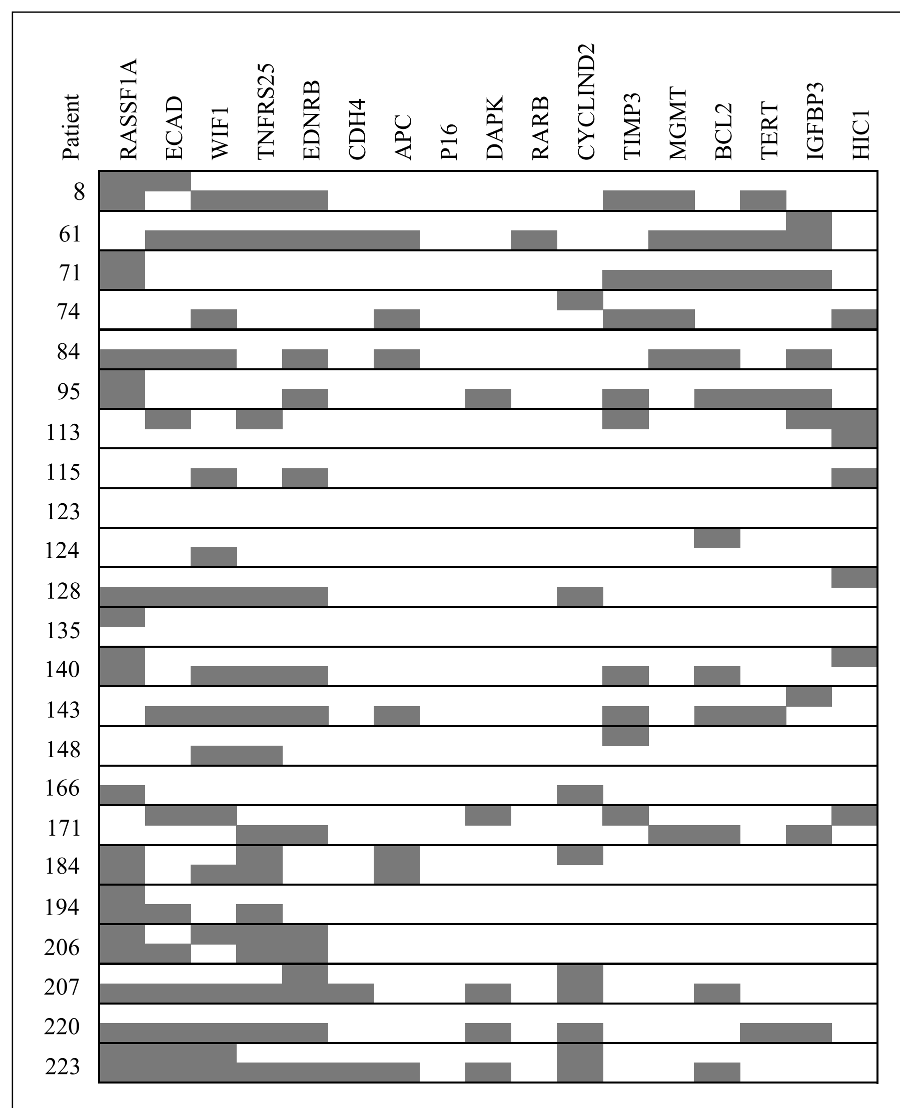

Fig. 1. Methylation at 17 loci in matched samples of normal and malignant urothelium. Gray boxes reveal the presence of methylation at each locus in the 23 patients with paired normal and malignant samples. For each patient, the top row is for normal DNA, and the bottom row is for malignant DNA analysis.

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12 (SPSS, Inc., Cary, NC). Categorical variables were compared using the χ^2 test, and continuous variables were analyzed with a t test. Recurrence, progression, and cancer-specific survival probabilities following tumor resection were analyzed using the Kaplan-Meier method and log-rank test. Multivariate analysis for predictors of tumor progression was done using Cox regression analysis. $P < 0.05$ was interpreted as statistically significant. Bonferroni's correction was used for multiple variable analyses.

We have previously found that artificial intelligence techniques improve the analysis of complex data and allows exploratory variable interrogation without dependence upon linearity (26). We developed Neuro-fuzzy models (NFM) to examine the importance of methylation at the various gene promoters. The models were developed within Matlab and are described in detail elsewhere (26). The data were divided into training (90%: 60% for training and 30% for validation) and testing cohorts (10%) and analyzed using cross-validation and ensembling techniques (10 batches). Two modeling strategies were used to rank the extent of each individual promoter's influence upon the predictive accuracy of NFM. A ''leave-one-out'' approach removed each variable in turn from the model, and a ''selectivity'' approach spanned each variable from minimum to maximum while fixing all the others within the model to nominal values. Following ranking, we used NFM to predict the presence and timing of progression, using either all 17 loci or a selected panel of loci (n = 5 loci). These models were developed in parallel to compare the predictive accuracy of using all 17 loci or just a selected panel. Cross-validation was used within each of these parallel models.

Results

Methylation in normal and tumor samples. Methylation was significantly more common in malignant than normal urothelium for 8 of 17 loci (Table 2; Figs. 1 and 2). Consequently, the methylation indices varied significantly between these two tissues (33.3% versus 10%; t test, P = 0.0001). The gene/β-actin ratio revealed that for seven loci, significantly higher concentrations of gene methylation were present in malignant tissues when compared with normal urothelium (Fig. 2). When tumor stage and grade were compared with methylation at the 17 loci, an increased frequency of methylation was seen in poorly differentiated than well-differentiated urothelial cancer (methylation index: 38% versus 28%; t test, P = 0.04) and in invasive than superficial tumors (Fig. 3; methylation index: 39% versus 32%; t test, P = 0.09).

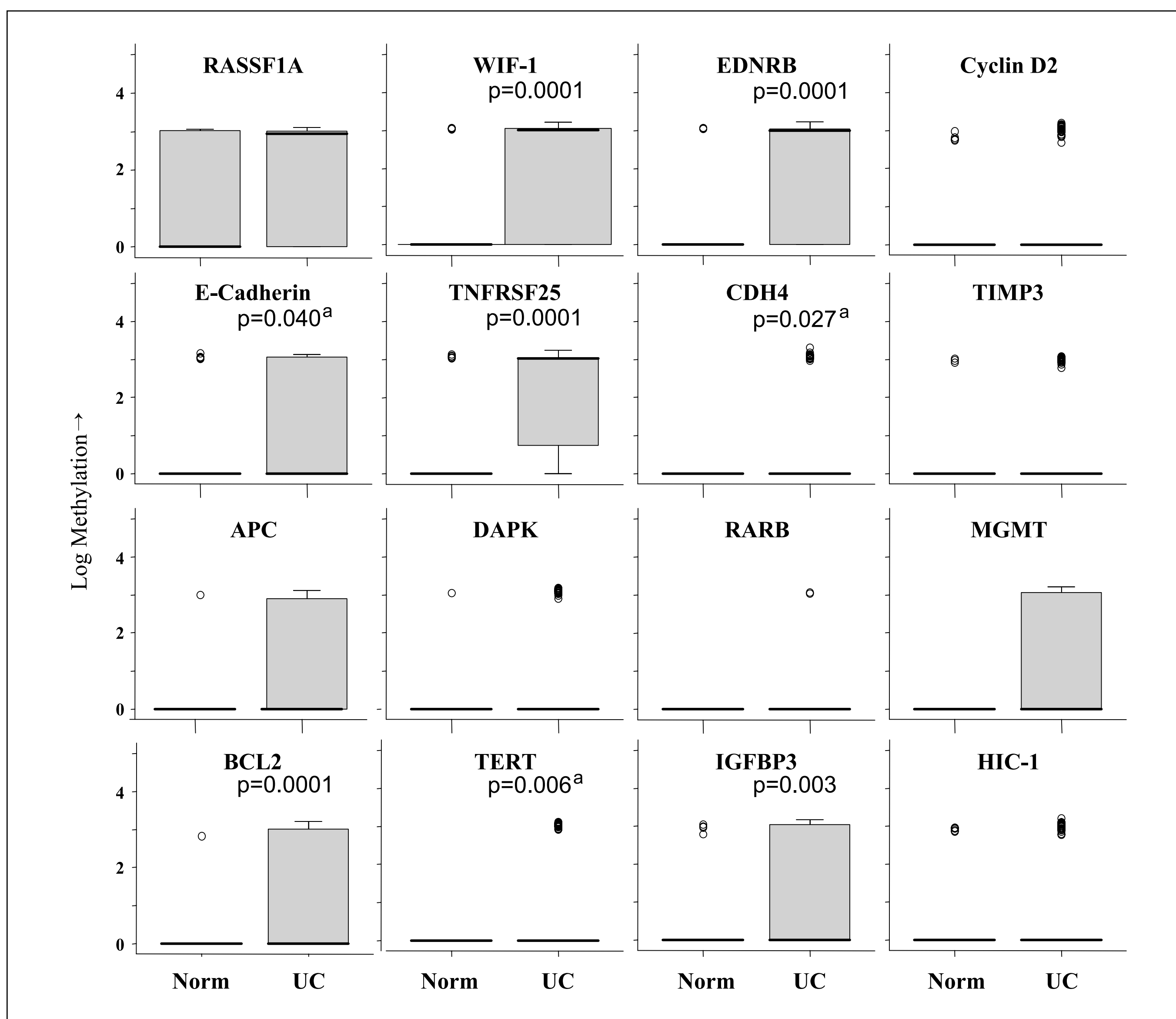

Fig. 2. Methylation concentrations in normal and urothelial cancer (*UC*) tissues. The box plots reveal the median (*thick line*), middle 50% (*box*), and 1.5 time interquartile range (*bars*) for the gene/β-actin ratio for both tissues. For diagrammatic simplicity, p16 is not shown (only one methylated sample, no difference found). For 7 of 17 loci, significantly higher concentrations of methylation were present in urothelial cancer than normal tissues (*t* test values). [a], not significant after Bonferonni's correction.

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Methylation as a prognostic marker. When tumor behavior was analyzed, in addition to tumor stage (log-rank, P = 0.0001) and grade (log-rank, P = 0.02), higher progression rates were seen in tumors with methylation of *E-cadherin* (log-rank, P = 0.00001), *TNFRSF25* (log-rank, P = 0.0016), *EDNRB* (log-rank, P = 0.0005), *RASSF1a* (log-rank, P = 0.02), and *APC* (log-rank, P = 0.05) than in tumors without methylation at these loci (Fig. 4A). Methylation at these loci was also significantly associated with an increased rate of subsequent patient death from urothelial cancer when compared with nonmethylated tumors (log-rank test: RASSF1a, P = 0.0009; E-cadherin, P = 0.039; TNFRSF25, P = 0.0028; EDNRB, P = 0.023). Cox multivariate analysis revealed that tumor stage (Cox hazard ratio, 1.949; 95% confidence interval, 1.346-2.822; P = 0.008), methylation of E-cadherin (Cox hazard ratio, 3.242; 95% confidence interval, 1.648-6.378; P = 0.02), and tumor methylation index (Cox hazard ratio, 18.116; 95% confidence interval, 2.768-118.554; P = 0.002) were independent predictive variables of tumor progression. None of the tumors with very low (≤1 of 17 methylated loci) or absent methylation subsequently progressed to a worse disease.

Analysis of prognostic panels. We have previously found that promoter methylation of the *RASSF1a*, *E-cadherin*, and *APC* genes were significantly associated with urothelial cancer progression (7, 14). Using this panel of three genes to stratify our tumors, there was significantly more progression in tumors with methylation of one or more loci than in those with no methylation (Fig. 4B; log-rank, P = 0.0020). Only 3 of 27 (11%) tumors with no methylation of these three genes subsequently progressed to worse disease [in contrast to 28 of 68 (41%) with methylation]. This panel has a sensitivity of 91%, specificity of 38%, and an AUROC of 64% for progression.

If we use a panel of the five loci most associated with progression (*E-cadherin*, *TNFRSF25*, *EDNRB*, *RASSF1a*, and *APC*) to define methylation, then those tumors with methylation of more than one locus have significantly higher progression rates (Fig. 4C; log-rank, $P = 0.0013$). This panel improves the sensitivity for progression (97% sensitivity, 38% specificity, and AUROC of 67%) by allowing the inclusion of those with a low level of methylation in the good prognosis group.

Artificial intelligence analysis. We developed and interrogated NFMs to analyze the 17 loci. Using the selectivity approach, the model ranked the top five loci in the same order as the log-rank test: *E-cadherin* (1st), *TNFRSF25*, *EDNRB*, *RASSF1a*, and *APC* (5th). Using this model, the NFM was able to predict the timing and presence of progression with 90% accuracy, 97% specificity, and 75% sensitivity. Using the leave-one-out approach, the NFM found that the loci that most influenced the model accuracy were *WIF-1*, *CDH4*, *hTERT*, and *RASSF1a*, in that order (93% accuracy, 100% specificity, and 78% sensitivity). We then used the trained models to predict the timing of tumor progression with either all 17 loci or just the five loci in our selected panel. The models did these predictions accurately and showed no difference in accuracy with the reduction of 17 to 5 loci (t test, $P = 0.9$).

Superficial tumors. One of the benefits of promoter methylation as a biomarker is its high sensitivity for the identification of patients at very low risk of tumor progression (18). For example, the presence of methylation could be used to identify those patients suitable for less frequent cystoscopic surveillance. If we analyze only the superficial urothelial cancer in this population (i.e., those undergoing cystoscopic surveillance),

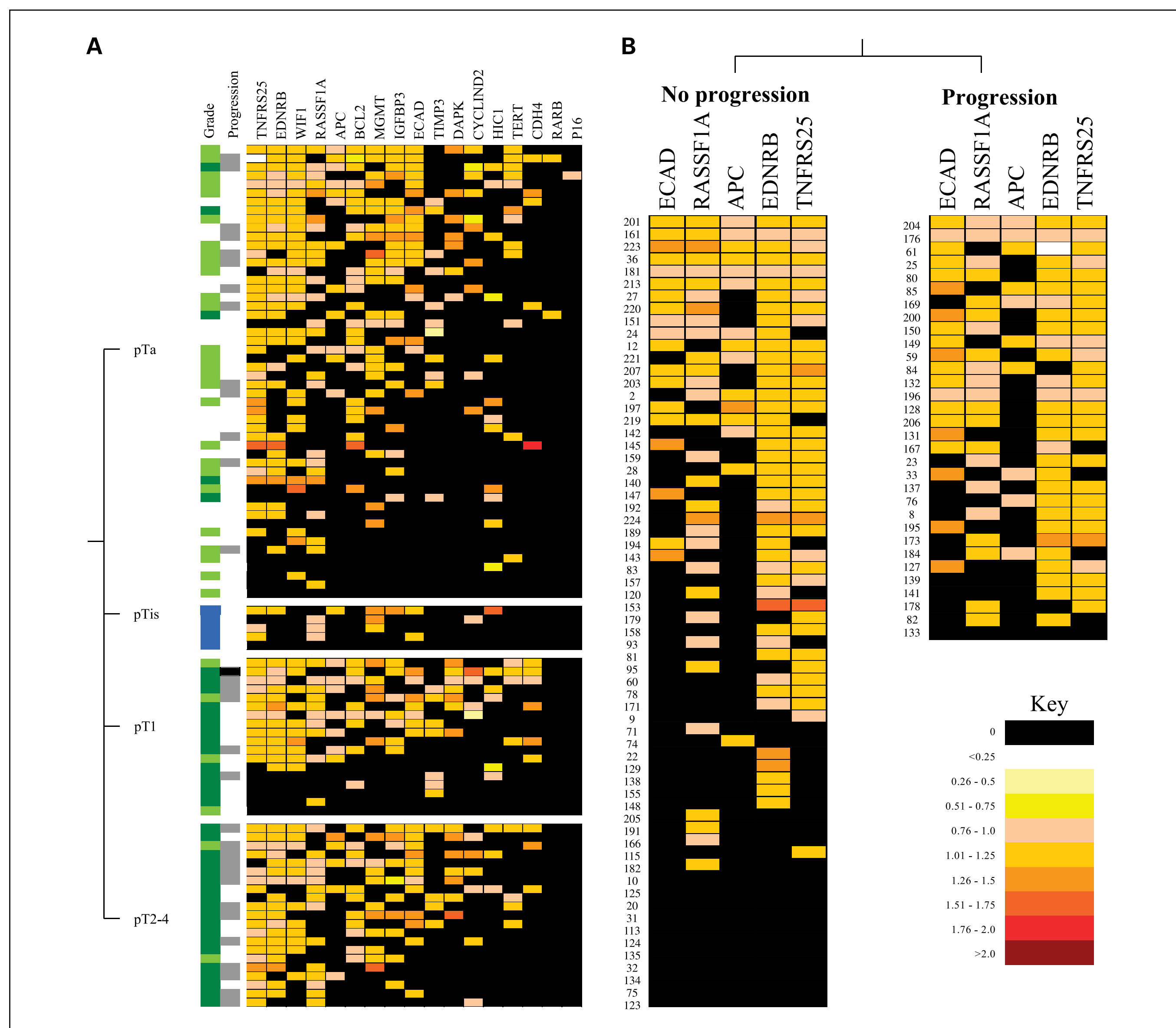

Fig. 3. *A,* heat map of quantitative methylated promoter gene/β-actin ratios in the 96 tumor samples stratified for stage. For each patient, the tumor grade (grade 1, *white*; grade 2, *light green*; grade 3, *dark green*; and carcinoma *in situ, blue*) and the presence of subsequent tumor progression (no progression, *white*; progression, *gray*; progression not known, *black*) are color coded. *B,* heat map of quantitative methylated promoter gene/β-actin ratios in the 96 tumor samples stratified for progression using the five-gene panel.

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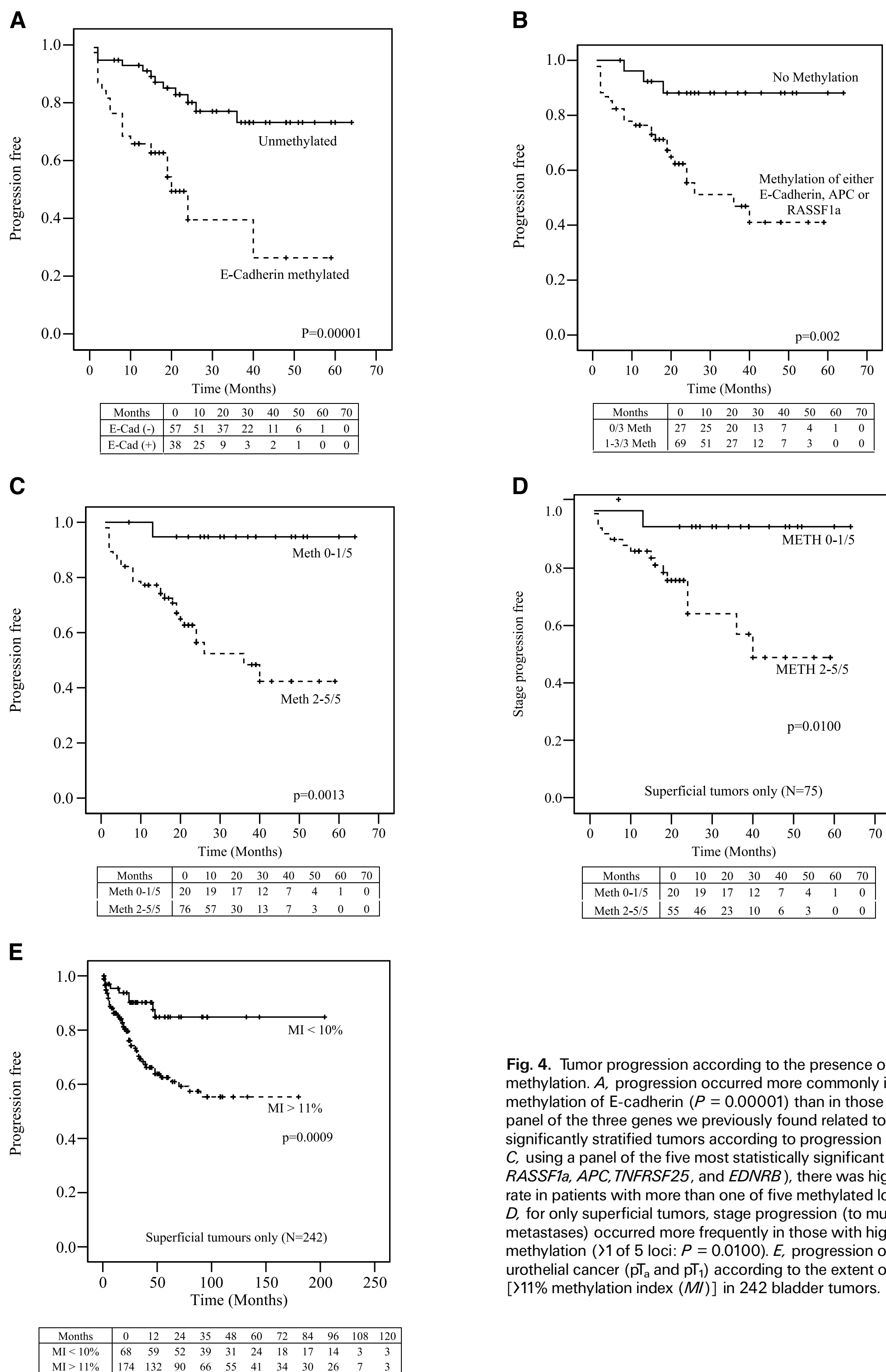

Months	0	10	20	30	40	50	60	70
E-Cad (-)	57	51	37	22	11	6	1	0
E-Cad (+)	38	25	9	3	2	1	0	0

Months	0	10	20	30	40	50	60	70
0/3 Meth	27	25	20	13	7	4	1	0
1-3/3 Meth	69	51	27	12	7	3	0	0

Months	0	10	20	30	40	50	60	70
Meth 0-1/5	20	19	17	12	7	4	1	0
Meth 2-5/5	76	57	30	13	7	3	0	0

Months	0	10	20	30	40	50	60	70
Meth 0-1/5	20	19	17	12	7	4	1	0
Meth 2-5/5	55	46	23	10	6	3	0	0

Months	0	12	24	35	48	60	72	84	96	108	120
MI < 10%	68	59	52	39	31	24	18	17	14	3	3
MI > 11%	174	132	90	66	55	41	34	30	26	7	3

Fig. 4. Tumor progression according to the presence of promoter methylation. *A,* progression occurred more commonly in tumors with methylation of E-cadherin ($P = 0.00001$) than in those without it. *B,* a panel of the three genes we previously found related to progression significantly stratified tumors according to progression ($P = 0.002$). *C,* using a panel of the five most statistically significant loci (*E-cadherin, RASSF1a, APC, TNFRSF25*, and *EDNRB*), there was higher progression rate in patients with more than one of five methylated loci ($P = 0.0013$). *D,* for only superficial tumors, stage progression (to muscle invasion or metastases) occurred more frequently in those with high levels of methylation (>1 of 5 loci: $P = 0.0100$). *E,* progression of superficial urothelial cancer (pT_a and pT_1) according to the extent of methylation [>11% methylation index (*MI*)] in 242 bladder tumors.

then methylation of E-cadherin (log-rank, $P = 0.002$) and the extent of methylation of the five-locus panel (Fig. 4D; log-rank, $P = 0.0100$) are significantly associated with tumor progression to muscle invasion or metastases.

Combining the data from our previous studies, we have now investigated the presence of promoter methylation in 480 urothelial cancer samples (7, 14). Of these, there are 242 new superficial tumors selected from patients with similar risk

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factors. If we stratify these according to level of methylation (methylation index ≤ 10%), those with infrequent methylation have significantly lower progression rates (Fig. 4E; log-rank, $P = 0.0009$) than those with frequent methylation. Of the 7 of 69 (12%) with a low frequency of methylation that progressed during follow-up, all were clinically suspicious for progression, having superficial invasion (pT_1) and moderate (grade 2, $n = 3$) or high-grade (grade 3, $n = 4$) differentiation. No progression to higher stage or grade was seen in the 149 pT_a tumors with a low level of methylation we have studied.

Discussion

Altered gene expression associated with promoter hypermethylation is recognized to be an important common event in cancer. The exact mechanism of gene silencing is unknown, as are the characteristics determining gene susceptibility to this mechanism of inactivation. For example, it is unclear why genes of similar function and mutational prevalence in hereditary syndromes vary in susceptibility to hypermethylation (e.g., *hMLH1* and *hMSH2* in colorectal cancer and *BRCA1* and *BRCA2* in breast cancer). Furthermore, there are differences in the pattern of gene methylation in tumors of different organs. Methylated genes can be divided into those that occur generally in most cancers (such as *E-cadherin* and *RASSF1a*), occur commonly in a few cancers (e.g., *APC* in bladder and colonic cancer), and those that are mostly specific to one organ (e.g., *GSTP1* in prostate cancer or *VHL* in renal cancer; ref. 27). Although the loci and extent of genes affected by hypermethylation vary between tumors, their loss reflects global changes in epigenetic control. By selecting loci for various tumors, one could devise organ-specific gene panels that reveal events across the entire epigenome. This knowledge is useful as epigenetic alterations affect the molecular mechanisms within a tumor and thus the subsequent clinical phenotype. For example, high levels of methylation are found in sporadic cancers with microsatellite instability due to hMLH1 methylation (6, 7). Tumors with this form of genomic instability are relatively chemoresistant and behave more indolently than matched tumors without microsatellite changes (28). Similarly, MGMT methylation alters sensitivity to temozolomide and could eventually be used to stratify patient treatment (29).

Here, we have studied 17 gene promoters in urothelial cancer, using a prospectively collected urothelial cancer biorepository. We confirmed that hypermethylation is present in histologically normal cells and increases with the onset of malignancy. As reported by Freidrich et al., we found several apoptosis related genes to be specific for malignant tissue and occur commonly enough in bladder cancer to appear clinically useful (15). Although some genes seemed to have strong individual associations with tumor progression (e.g., *E-cadherin*), we were keen to develop a panel of loci that would be applicable to all urothelial cancer, be specific for cancer, and represent the extent of global epigenetic disruption. We initially used the three-gene panel that our previous work had suggested (*E-cadherin*, *RASSF1a*, and *APC*). This panel successfully stratified tumors according to progression (Fig. 4B). Although 28% of tumors had no methylation according to this panel, there were three tumors that progressed to worse disease in the good prognosis cohort. When *TNFRSF25* and *EDNRB* (the other loci associated with progression) were added to create a five-locus panel, the larger number of genes allowed sample discrimination according to the extent (high, 2 – 5/5 or low, 0 – 1/5 levels) of methylation, rather than just its presence or absence. We feel it is important to allow minimal levels of methylation within a good prognostic group, as methylation can be detected at low levels within normal epithelia. This five-locus panel improved tumor discrimination (Fig. 4C) and produced a low-risk group with 21% of all cases and only one tumor progression (a grade 3, pT_1 tumor). In particular, this panel was able to discriminate superficial cancer progression to a more advanced stage (Fig. 4D). Artificial intelligence analysis of our results confirmed the ranking of our top five loci and revealed that predictions using a panel of these five loci were as accurate as those made from data using all 17 loci (data not shown).

Combining the data from our previous studies, we have now investigated the presence of promoter methylation in 480 urothelial cancer samples (7, 14). In each population, we found the presence of methylation to be a strong predictor of tumor progression and death from urothelial cancer. If we focus on the use of this marker to identify low-risk disease and look at only superficial cancers (where reduced frequency cystoscopic surveillance is an option), then it seems to robustly identify a cohort (around one of three of new patients) with a low progression risk (Fig. 4D and E). Those tumors with favorable pathologic features and a low methylation index could be surveyed less frequently to reduce morbidity and cost.

Our results confirm the prognostic importance of gene methylation as reported in various tumors. For example, Roman-Gomez et al. used a candidate loci approach (with 27 gene promoters) in acute lymphoblastic leukemia and found that the absence of the methylator phenotype (in 32% of 38 patients) was associated with a good prognosis (18). Wei et al. used 12,000 CpG island arrays to analyze the global patterns of methylation in ovarian tumors and identified differentially methylated loci that could accurately predict progression (30). These reports support our conclusion that selected small loci panels can be developed to reflect the global epigenetic state in a tumor. As different patterns of gene methylation are present in different organs, it is likely that organ-specific progression panels will be required. As many of these epigenetic alterations can be detected in excreted bodily fluids [such as urine (16) and saliva (31)], it is possible that prognostic panels could be used within the office setting.

In summary, the presence of frequent gene promoter methylation is a poor prognostic sign in urothelial cancer, independent of tumor stage or grade. In superficial disease, those with low or absent methylation could be followed with less intensive cystoscopic surveillance, and the risk of tumor progression is low. Organ-specific panels of loci could be developed that reflect global epigenetic events and would be small enough to be practical for routine application. Further multi-institutional studies are required to validate this marker and its general applicability in the management of patients with urothelial cancer.

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