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A DNA hypermethylation profile reveals new potential biomarkers for the evaluation of prognosis in urothelial bladder cancer

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López JI, Angulo JC, Martín A, Sánchez-Chapado M, González-Corpas A, Colás B, Ropero S. A DNA hypermethylation profile reveals new potential biomarkers for the evaluation of prognosis in urothelial bladder cancer. APMIS 2017.

DNA hypermethylation has emerged as a molecular biomarker for the evaluation of cancer diagnosis and prognosis. We define a methylation signature of bladder cancer and evaluate whether this profile assesses prognosis of patients. Genome-wide methylation analysis was performed on 70 tumor and 10 normal bladder samples. Hypermethylation status of 1505CpGs present in the promoter region of 807 genes was studied. Thirty-three genes significantly hypermethylated in ≥10% of the tumors. Three clusters of patients were characterized by their DNA methylation profile, one at higher risk of dead of disease (p = 0.0012). Association between cluster distribution and stage (p = 0.02) or grade (p = 0.02) was demonstrated. Hypermethylation of *JAK3* and absence of hypermethylation of *EYA4*, *GAT6*, and *SOX1* were associated with low-grade non-invasive disease. On the other hand, in high-grade invasive disease hypermethylation of *CSPG2*, *HOXA11*, *HOXA9*, *HS3ST2*, *SOX1*, and *TWIST1* was associated with muscle invasiveness. A panel of hypermethylated genes including *APC*, *CSPG2*, *EPHA5*, *EYA4*, *HOXA9*, *IPF1*, *ISL1*, *JAK3*, *PITX2*, *SOX1*, and *TWIST1* predicted cancer-specific survival and *SOX1* (HR = 3.46), *PITX2* (HR = 4.17), *CSPG2* (HR = 5.35), and *JAK3* hypermethylation (HR = 0.19) did so independently. Silencing of genes by hypermethylation is a common event in bladder cancer and could be used to develop diagnostic and prognostic markers. Combined hypermethylation of *SOX1*, *PITX2*, or *CSPG2* signals patients at higher risk of death from bladder cancer.

Key words: Bladder cancer; DNA methylation; biomarkers; epigenetics; disease-specific survival.

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Spain has one of the highest incidences of bladder cancer in Europe despite the efforts displayed to control smoking habit, with a 4.6:1 male-to-female ratio (1). This gender difference in disease incidence is independent of differences in exposure risk, including smoking status. Bladder cancer mortality is associated with distinct environmental and socioeconomic factors, with their effects varying by region, race, and gender (2). Estimations reveal mortality of the disease will increase between 1998 and 2022 in Spain (3).

The identification of specific and sensitive molecular biomarkers in the fields of genomics, proteomics, and epigenetics has exponentially increased but disease detection and surveillance remains dependent on invasive procedures, mainly cystoscopy, and no validated biomarker currently exists in routine clinical practice other than cytology (4). Bladder cancer analysis by DNA microarrays provides new putative mRNA markers for bladder cancer diagnosis and prognosis that can be

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extrapolated to bladder fluids (5). Extensive investigation has been performed to detect new molecular biomarkers and better predict outcome. Epigenetic alterations have been considered promising tools for cancer diagnosis, evaluation of prognosis, and treatment response.

DNA methylation changes have been reported in bladder cancer, and many of them correlate with tumor grade and invasiveness (6–8). Also, identification and validation of novel methylated genes and their application as urinary tumor markers is a field of increasing interest (9, 10). DNA promoter hypermethylation is linked to gene silencing and a common event in bladder cancer development affecting genes involved in key cellular functions like cell cycle control, transcription, cell–cell adhesion, apoptosis, cell differentiation, and epithelial–mesenchymal transition (10). Promoter hypermethylation of *RASSF1a*, *E-cadherin*, *TNFSR25*, *EDNRB*, and *APC* identify progression risk in bladder cancer (11). Also, DNA methylation profiling may provide optimal indicator for carcinogenetic risk estimation (12). On the other hand, hypomethylation within gene bodies is usually associated with upregulated expression (13). Current methods for the determination of DNA methylation allow rapid and accurately identification of epigenetic modifications, both in clinical specimens and in body fluids.

We defined an epigenetic signature to evaluate patients with prostate cancer (14, 15). Some of these findings could have important implications to establish optimum responders to different treatment regimens. We also studied whether a gene hypermethylation profile could provide a specific signature of bladder cancer in our environment and discriminate populations of patients at different risk of mortality from the disease.

MATERIAL AND METHODS

Patient population

Aged-matched normal bladder tissue from organ donors at brain death (n = 10) and a series of patients with urothelial carcinoma (n = 70) was obtained (Table 1). Formalin-fixed and paraffin-embedded (FFPE) tissue blocks were retrieved. Representative regions with >90% tumor were selected on hematoxylin and eosin (H&E)-stained sections and punch biopsies were taken from the corresponding FFPE blocks for DNA and RNA extraction.

Patients selected were a single-institution longitudinal cohort. They all were treated in the same academic institution for a 10-year period so that follow-up was available. Cases were selected under several premises: (i) tissue was abundant and devoid of coagulation artifact; (ii) mayor clinical groups of disease (non-invasive, non-muscle invasive, and muscle invasive) were similarly balanced; (iii) no patient received neoadyuvant systemic chemotherapy or intravesical instilation before specimen retrieval to avoid treatment artifacts; (iv) urothelial cancer variants other than TCC were not included; (v) primary carcinoma *in situ* was also excluded.

Table 1. Patient characteristics

Clinical parameter	n (%)
Age, years	
≤70	23 (32.9)
>70	47 (67.1)
Sex	
Male	59 (84.3)
Female	11 (15.7)
Focality of tumors	
Single	21 (30)
Multiple	49 (70)
Active smoking habit	
No	33 (47.1)
Yes	17 (24.3)
NA	20 (28.6)
Urine citology	
Negative	47 (67.1)
Positive	23 (32.9)
Grade (WHO 1994)	
Grade 1	18 (25.7)
Grade 2	12 (17.1)
Grade 3	40 (57.1)
Grade (WHO/USIP 2004)	
Low grade	20 (28.6)
High grade	50 (71.4)
T category	
Non-invasive (Ta)	27 (38.6)
Non-muscle invasive (T1)	22 (31.4)
Muscle-invasive (≥T2)	21 (30)
Associated cis	
No	22 (37.9)
Yes	33 (56.9)
Progression to metastases	
No	52 (75)
Yes	18 (25)
Dead of bladder cancer	
No	54 (77.1)
Yes	16 (22.9)

NA, non available.

Radical cystectomy was performed as treatment in 18 patients and included extensive lymph node dissection. In three cases with muscle-invasive disease, cystectomy was not performed because they were openly metastatic at diagnosis. Patients with non-invasive or non-muscle invasive disease (n = 49) received transurethral resection (TUR) and intravesical instillation of chemotherapy or BCG according to standard protocols regarding EORTC risk stratification. All patients were periodically followed up for tumor recurrence or progression. The primary endpoint assessed in this study was cancer-specific survival (CSS). Secondary endpoint was progression to metastases.

Paraffin-embedded blocks with abundant malignant tissue were identified. TUR samples were selected to avoid coagulation artifact. Confirmation of urothelial carcinoma and evaluation of histological grade and the level of bladder wall invasion were defined after complete agreement with double pathologist review. DNA extraction from

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paraffin-embedded tissues was performed according to standard protocols. DNA was also obtained from 10 normal prostate tissue donated at the time of organ explants from male adults at brain death. Age of controls was matched with that of patients with bladder urothelial carcinoma. The study was approved by local Ethics Committee.

DNA methylation analysis

A golden gate methylation Cancer pannel (Illumina) was used to quantify DNA methylation on 70 bladder cancer specimens and 10 normal bladder tissues. The panel interrogated for the methylation state of 1505 CpGs sites selected from 807 cancer-related genes. Methylation assay was performed and four probes were designed for each CpG site: two allele-specific oligos (ASOs) and two locus-specific oligos (LSOs). Each ASO-LSO oligo pair corresponded to either the methylated or unmethylated state of the CpG site. Bisulfite conversion of DNA samples was done using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). The array was hybridized under a temperature gradient program, and arrays were imaged using a BeadArray Reader (Illumina Inc.). Image processing and intensity data extraction software were used. Each methylation data point is represented by fluorescent signals from the M (methylated) and U (unmethylated) alleles. Background intensity computed from a set of negative controls was subtracted from each analytical data point. The beta value was then calculated as the ratio of fluorescent signals from the two alleles according to the following formula: $\beta\text{-value} = [\mathrm{Max}(M,0)]/[\mathrm{Max}(U,0) + \mathrm{Max}(M,0) + 100]$. The beta value is a quantitative measure of DNA methylation levels of every CpG included in the array, and ranges from 0 (completely unmethylated) to 1 (completely methylated).

Hierarchical clustering was performed on all 70 cases and 10 control tissues using the cluster analysis tool of the BeadStudio Software (version 3.2). Patient methylation profile in selected genes was determined by non-supervised segmentation, applying a cluster analysis (Ward method), over the non-standardized methylation information of patients.

Statistical analysis

Mann–Whitney *U*-test, the Chi-square contingency test and Fisher's exact test were performed to compare differences between data, depending on scale of measure of evaluated variables. Holm correction was used for multiple comparisons to avoid false discovery rate. Survival analysis was performed using Kaplan–Meier method with significance evaluated by two-sided log-rank test (at a level of 0.1). Cox regression was performed using a stepwise model with a threshold entry $p = 0.15$ and stay criterium $p = 0.20$. The statistical analysis was developed using JMP9.0.2 and SAS9.3 (202-2010 by SAS Institute Inc., Cary, NY, USA).

RESULTS

DNA hypermethylation profile of bladder cancer samples

Patient characteristics are described in Table 1. Mean age was 68.5 ± 10.1 years (34–88). At a mean follow-up of 57.1 months (95% CI: 49.3–64.9, range 26–136), 16 patients (22.9%) died of disease. Thereof, disease-specific survival was 94.3%, 79.1%, and 71.9% at 1, 5, and 10 years. Unsupervised hierarchical cluster analysis showed that normal tissues shared a common DNA hypermethylation profile different from bladder cancer (Fig. 1). The DNA methylation profile of tumor samples was heterogeneous, ranging from very similar to normal tissues to others with great differences probably reflecting clinico-pathological diversity of the disease.

To identify genes methylated in tumors samples we selected those probes unmethylated in normal tissues (β-value < 0.2) and methylated in tumor samples (β-value > 0.5). This analysis provided a list of 195 probes hypermethylated in at least one tumor sample. We have considered only 40 probes corresponding to 33 genes because they were significantly hypermethylated in at least 10% of the tumors analyzed ($p < 0.05$) (Table 2). Hypermethylation of *HOXA9*, *SOX1*, *CCNA1*, *APC*, *WT1*, and *TWIST1* was present in 58.6%, 24.3%, 21.4%, 20%, 18.6%, and 18.6% of the cases analyzed, respectively. More interestingly, we identified new hypermethylated genes including *HOXA11*, *PENK*, *CYP1B1*, *EPHA5*, *JAK3*, and *CDH11*, hypermethylated in 60%, 48.6%, 41.4%, 37.1%, 32.9%, and 30% of the cases in this series, respectively.

DNA hypermethylation profile and clinico-pathological features

Since the samples studied came from a variety of patients with non-invasive and invasive disease, the relationship between the hypermethylation frequency of selected genes and clinico-pathological features of the patients was investigated. The methylation profile of low-grade, non-invasive high-grade, and invasive high-grade disease was compared. Hypermethylation of *JAK3* and absence of hypermethylation of *EYA4*, *GAT6*, and *SOX1* were associated ($p \leq 0.05$) with low-grade non-invasive disease (Table 3). On the other hand, hypermethylation of *GATA6* was more frequent in non-invasive than in invasive high-grade disease, but this difference did not reach statistical significance (30.8% vs 10.8%; $p < 0.18$). Regarding high-grade invasive disease, hypermethylation of *CSPG2*, *HOXA11*, *HOXA9*, *HS3ST2*, *SOX1*, and *TWIST1* was more frequent ($p \leq 0.05$) in muscle-invasive disease than in tumors invading lamina propia (Table 4).

Hierarchical clustering analysis identified three clusters of patients according to their gene hypermethylation profile (Fig. 2). Analysis of clinico-pathological features revealed differences between clusters. Patients in cluster 1 presented determinants of worse

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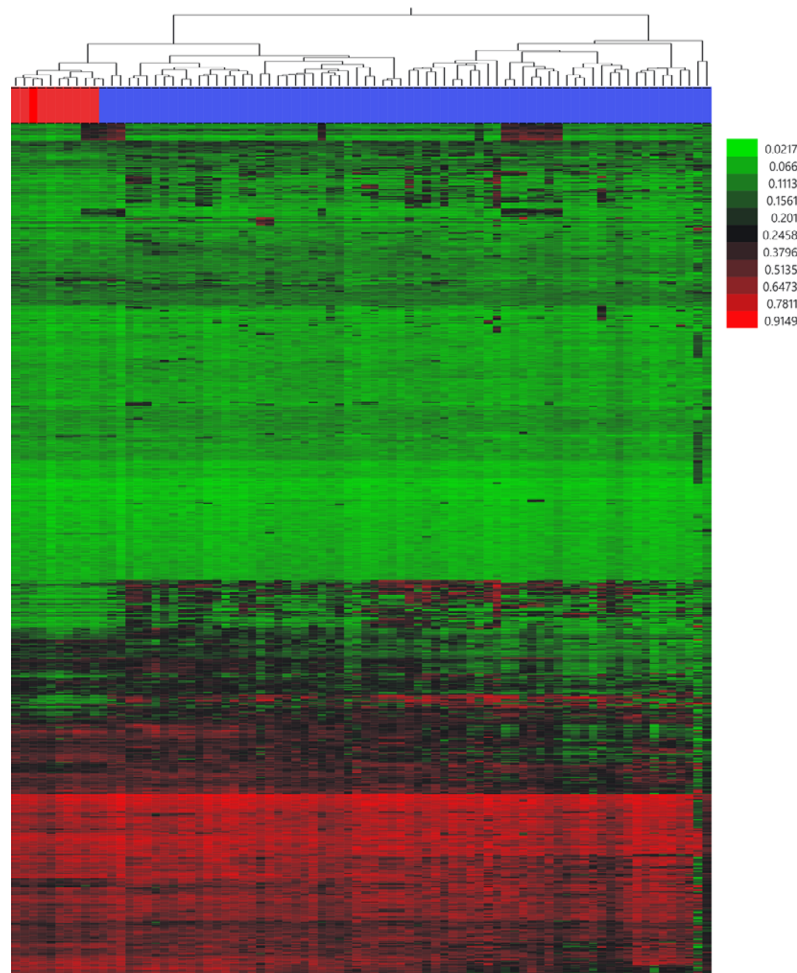

Fig. 1. Hierarchical cluster analysis of DNA methylation. Data extracted from 70 DNA samples of paraffin-embedded bladder cancer (blue) and 10 normal bladder tissues (red).

prognosis; such as muscle invasive disease and high-grade tumor predominance. High-grade tumors were also more frequent in cluster 2. Tumors in cluster 3 more often were non-invasive (Table 5). Regarding other clinical features, older patients presented more often in clusters 1 and 3 and active smokers in cluster 2; however, these trends were not significant (Table 5). Also, patients included in a cluster were characterized according to the frequency of hypermethylation for each individual gene; that is, a gene differentially methylated in a group of patients and not in the rest was a good definer of the cluster (Table 6). All genes identified except for *FZD9*, *GSTM1*, *JAK3*, and *NEFL* appeared methylated at higher degree in cluster 1.

DNA hypermethylation profile and prognosis

Significant differences in disease-specific survival were evidenced among the clusters (log-rank, p = 0.0012) (Fig. 2). Differences regarding the interval free of metastatic progression were also confirmed (log-rank, p = 0.0024). These findings suggested that the DNA hypermethylation profile could segregate patients with different metastatic potential.

We aimed to define the methylation signature that could predict CSS and found patients included in cluster 1 were at higher risk of death of disease than patients in cluster 2 (HR = 2.37, 95% CI: 0.82–6.86) and cluster 3 (HR = 7.2, 95% CI: 1.52–34.42) indicating that the gene hypermethylation profile defining cluster 1 could be a good predictor of metastatic development and subsequent patient death. Patients in cluster 2 also were at higher risk of death than those in cluster 3 (HR = 3.06, 95% CI: 0.62–15.18).

Univariate analysis for CSS according to the methylation status of each different gene identified hypermethylation of *APC* (HR = 2.97, 95% CI:

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Table 2. List of genes significantly hypermethylated in at least 10% of the bladder tumors analyzed

Probes	Genes	Annotation	%	p-value
HOXA11_P698_F	HOXA11	Homeobox A11	60.0	1.1E-05
HOXA9_P1141_R	HOXA9	Homeobox A9	58.6	1.25E-05
PENK_E26_F	PENK	Proenkephalin	48.6	7.15E-06
CYP1B1_E83_R	CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	41.4	1.0E-03
EPHA5_P66_F	EPHA5	EPH receptor A5	37.1	5.1E-06
JAK3_P156_R	JAK3	Janus kinase 3	32.9	1.1E-05
EYA4_E277_F	EYA4	Eyes absent homolog 4	31.4	1.9E-05
TAL1_P594_F	TAL1	T-cell acute lymphocytic leukemia 1	31.4	6.7E-06
PITX2_E24_R	PITX2	Paired-like homeodomain 2	31.4	9.0E-05
CDH11_P354_R	CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	30.0	4.3-06
SOX1_P294_F	SOX1	SRY (sex determining region Y)-box 1	24.3	3.0-05
NPY_P295_F	NPY	Neuropeptide Y	21.4	2.0-05
GSTM2_E153_F	GSTM2	Glutathione S-transferase mu 2	21.4	3.0E-04
CCNA1_E7_F	CCNA1	Cyclin A1	21.4	9.0E-04
APC_P14_F	APC	Adenomatous polyposis coli	20.0	5.0E-03
WT1_P853_F	WT1	Wilms tumor 1	18.6	2.5E-05
TWIST1_E117_R	TWIST1	Twist family bHLH transcription factor 1	18.6	3.0E-02
HS3ST2_E145_R	HS3ST2	Heparan sulfate (glucosamine) 3-O-sulfotransferase 2	18.6	3.0E-04
GSTM1_P266_F	GSTM1	Glutathione S-transferase mu 1	18.6	2.2E-05
ESR1_P151_R	ESR1	Estrogen receptor 1	18.6	1.55E-05
ATP10A_P147_F	ATP10A	ATPase, class V, type 10[a]	18.6	8.2E-05
FZD9_E458_F	FZD9	Frizzled class receptor 9	17.1	2.8E-04
CSPG2_P82_R	CSPG2	Versican	17.1	3.0E-04
BDNF_P259_R	BDNF	Brain-derived neurotrophic factor	17.1	4.1E-05
DCC_P471_R	DCC	DCC netrin 1 receptor	15.7	5.1E-05
SOX17_P287_R	SOX17	SRY (sex determining region Y)-box 17	14.3	1.6E-05
NEFL_E23_R	NEFL	Neurofilament, light polypeptide	12.9	4.0E-04
ISL1_P554_F	ISL1	ISL LIM homeobox 1	12.9	5.1E-05
IPF1_P234_F	IPF1	Pancreatic and duodenal homeobox 1	12.9	2.3E-02
FLT3_E326_R	FLT3	fms-Related tyrosine kinase 3	12.9	5.1E-05
CDH13_P88_F	CDH13	Cadherin 13, H-cadherin	12.9	6.7E-05
GATA6_P726_F	GATA6	GATA-binding protein 6	11.4	2.0-04
TMEFF2_P152_R	TMEFF2	Transmembrane protein with EGF-like and two follistatin-like domains 2	10.0	7.4E-05

Table 3. Differential hypermethylation profile between non-invasive low-grade, non-invasive high-grade, and invasive high-grade bladder cancer

Genes	Non-invasive low-grade, n = 14	Non-invasive high-grade, n = 13	Invasive high-grade, n = 37	p-value*
EYA4	0 (0%)	3 (27%)	16 (43.2%)	0.005
GATA6	0 (0%)	4 (30.8%)	4 (10.8%)	0.05
JAK3	8 (57.1%)	3 (23.1%)	8 (21.6%)	0.04
SOX1	0 (0%)	3 (23.1%)	12 (32.4%)	0.035

*Fisher exact test; six patients with invasive low-grade disease not included.

Table 4. Differential hypermethylation profile between high-grade non-muscle invasive, and muscle-invasive bladder cancer

Genes	Non-muscle invasive high-grade disease, n = 16	Muscle invasive high-grade disease, n = 21	p-value*
CSPG2	1 (6.25%)	8 (38.1%)	0.05
HOXA11	6 (37.5%)	15 (71.4%)	0.04
HOXA9	7 (43.75%)	16 (76.2%)	0.045
HS3ST2	1 (6.25%)	8 (38.1%)	0.05
SOX1	1 (6.25%)	11 (52.4%)	0.003
TWIST1	1 (6.25%)	8 (38.1%)	0.05

*Fisher exact test.

1.07–8.24), *CSPG2* (HR = 3.24, 95% CI: 1.17–8.92), *EPHA5* (HR = 2.49, 95% CI: 0.92–6.7), *EYA4* (HR = 2.41, 95% CI: 0.9–6.43), *HOXA9* (HR = 2.49, 95% CI: 0.8–7.73), *IPF1* (HR = 3.05, 95% CI: 0.84–11.12), *ISL1* (HR = 3.38, 95% CI: 0.95–11.97), *JAK3* (HR = 0.29, 95% CI: 0.06–1.26),

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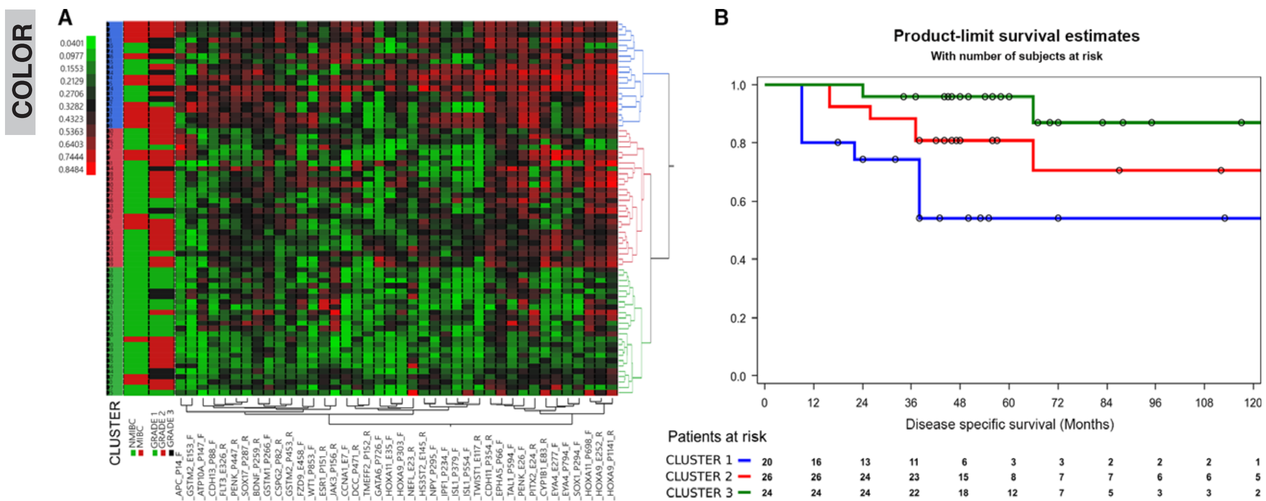

Fig. 2. (A) Hierarchical clustering of methylation values of the 40 significantly methylated probes in all prostate cancer samples. The dendrogram identifies three groups of patients with homogeneous DNA methylation profile: cluster 1 (blue), cluster 2 (red), and cluster 3 (green). (B) Kaplan–Meier curves for cancer-specific survival according to the clusters identified (log-rank, $p < 0.0012$).

Table 5. Comparison of the clinico-pathological features (%) of the patients included in each cluster

Variables	Cluster 1, n = 20	Cluster 2, n = 26	Cluster 3, n = 24	p-value*
T category				
Non-invasive (Ta)	15	38.5	58.3	0.02
Non-muscle invasive (T1)	30	34.6	29.2	
Muscle-invasive (>T2)	55	26.9	12.5	
Grade (WHO 1994)				
Grade 1	10	19.2	45.8	0.02
Grade 2	30	7.8	16.7	
Grade 3	60	73.1	37.5	
Grade category (WHO/USIP 2014)				
Low	20	19.2	45.8	0.06
High	80	80.8	54.2	
Patient age (years)				
≤70	20	46.2	29.2	0.15
>70	80	53.9	70.8	
Patient sex				
Male	90	76.9	87.5	0.54
Female	10	23.1	12.5	
Active smoking habit[1]				
No	75	52.2	78.9	0.16
Yes	25	47.8	21.1	

*Cochran Armitage test.
[1]Data for assessment of smoking habit available in 50 patients.

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PITX2 (HR = 3.24, 95% CI: 1.2–8.72), *SOX1* (HR = 3.46, 95% CI: 1.28–9.35), and *TWIST1* (HR = 2.78, 95% CI: 0.96–8.05) were predictors of prognosis in this series (Fig. 3). The correlation between the methylation status of these genes ranged from −0.17 (*JAK3* & *IPF1*) to 0.70 (*TWIST1* & *ISL1*). Multivariate analysis revealed hypermethylation of *SOX1* (HR3.46, 95% CI: 1.28–9.35), *PITX2* (HR4.17, 95% CI: 1.46–11.9), *CSPG2* (HR5.35, 95% CI: 1.75–16.1), and *JAK3* (HR0.19, 95% CI: 0.04–0.89) were individual predictors of CSS in this population of patients, the last marker behaved as a protector. Combined hypermethylation of at least two of the three genes that had a negative impact on prognosis (*SOX1*, *PITX2*, or *CSPG2*) occurred in 13/70 patients in the series (18.6%), 8 of which (61.5%) died of disease during follow-up. This hypermethylation signature followed a very dismal prognosis in this series with 35.9% (95% CI: 11.7–61.3) 5-year CSS, compared

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Table 6. Differences in the frequency (%) of hypermethylation for each individual gene among clusters: 29 genes are differentially methylated and 14 genes show significantly higher methylation in cluster 1 than in cluster 2

Genes	Cluster 1, n = 20	Cluster 2, n = 26	Cluster 3, n = 24	p-value*, (all clusters)	p-value**, (cluster 1 vs 2)
APC	8 (40%)	6 (23.1%)	0 (0%)	**1.5E-03**	0.99
ATP10A	8 (40%)	1 (3.85%)	4 (16.7%)	**8.0E-03**	**1.7E-02**
BDNF	8 (40%)	2 (7.7%)	2 (8.3%)	**1.2E-02**	**3.7E-02**
CCNA1	11 (55%)	4 (15.4%)	0 (0%)	**1.0E-04**	**2.9E-02**
CDH11	12 (60%)	8 (30.8%)	1 (4.2%)	**2.0E-04**	0.21
CDH13	6 (30%)	2 (7.7%)	1 (4.2%)	**4.0E-02**	0.19
CSPG2	9 (45%)	3 (11.5%)	0 (0%)	**2.0E-04**	5.1E-02
CYP1B1	12 (60%)	16 (61.5%)	1 (4.2%)	**4.0E-02**	1
DCC	8 (40%)	3 (11.5%)	0 (0%)	**6.0E-04**	0.11
EPHA5	15 (75%)	9 (34.6%)	2 (8.3%)	**1.0E-04**	**2.6E-02**
ESR1	8 (40%)	1 (3.85%)	4 (16.7%)	**8.0E-03**	**1.8E-02**
EYA4	12 (60%)	9 (34.6%)	1 (4.2%)	**2.0E-04**	0.4
FLT3	7 (35%)	1 (3.85%)	1 (4.2%)	**3.2E-03**	**4.2E-02**
FZD9	5 (25%)	5 (19.2%)	2 (8.3%)	0.34	1
GATA6	4 (20%)	4 (15.4%)	0 (0%)	**6.2E-02**	1
GSTM1	4 (20%)	6 (23.1%)	3 (12.5%)	0.64	1
GSTM2	7 (35%)	7 (26.9%)	1 (4.2%)	**2.4E-02**	1
HOXA11	17 (85%)	18 (69.2%)	7 (29.2%)	**4.0E-04**	0.9
HOXA9	17 (85%)	19 (73%)	5 (20.8%)	**1.0E-05**	1
HS3ST2	11 (55%)	2 (7.7%)	0 (0%)	**1.0E-05**	**2.0E-03**
IPF1	9 (45%)	0 (0%)	0 (0%)	**1.0E-05**	**4.5E-04**
ISL1	8 (40%)	1 (3.85%)	0 (0%)	**2.0E-04**	**1.8E-02**
JAK3	9 (45%)	6 (23.1%)	8 (33.3%)	0.31	0.61
NEFL	3 (15%)	2 (7.7%)	4 (16.7%)	0.66	1
NPY	11 (55%)	4 (15.4%)	0 (0%)	**1.0E-05**	**2.9E-02**
PENK	17 (85%)	13 (50%)	4 (16.7%)	**1.0E-05**	**8.2E-02**
PITX2	11 (55%)	7 (26.9%)	4 (16.7%)	**2.2E-02**	0.21
SOX17	7 (35%)	1 (3.85%)	2 (8.3%)	**9.0E-03**	**4.3E-02**
SOX1	13 (65%)	4 (15.4%)	0 (0%)	**1.0E-05**	**2.4E-03**
TAL1	13 (65%)	6 (23.1%)	3 (12.5%)	**7.0E-04**	**1.9E-02**
TMEFF2	6 (30%)	1 (3.85%)	0 (0%)	**2.5E-03**	0.1
TWIST1	13 (65%)	0 (0%)	0 (0%)	**1.0E-05**	0
WT1	8 (40%)	3 (11.5%)	2 (8.3%)	**2.6E-02**	0.11

*Fisher exact test.
**Fisher exact test corrected by Bonferroni; statistically significant differences (p < 0.05) in bold characters.

to 88.9% (95% CI: 76.9–94.9) if none or one appeared hypermethylated (log-rank, $p < 0.0001$) (Fig. 3).

DISCUSSION

A great number of studies try to identify genetic and epigenetic biomarkers for bladder cancer diagnosis and prognosis. Among the difficulties, DNA methylation is an epigenetic modification that can be affected by age, diet, and environment exposure (16) and also can be detected in non-malignant tissue in vicinity of neoplasia (17). To make matters worse, distinct DNA methylation epigenotypes in the same disease may also vary from different geographical populations (18). Finally, use of single-gene hypermethylation for bladder cancer diagnosis and prediction of prognosis may produce unspecific results since it may be derived from other occult neoplasms and also from non-malignant tissues. However, important efforts have been directed to analyze the diagnostic and prognostic value of individual hypermethylated genes.

Most methylation studies initially focused on single genes commonly methylated in bladder cancer and some years later genome-wide studies were performed in patients with bladder cancer using different approaches. These studies identified a number of silenced genes by promoter methylation in bladder cancer some of which were proposed as molecular markers for bladder cancer diagnosis and prognosis. Our results reproduced DNA methylation at specific loci already described by several authors, including *HOXA9* (9, 17, 19), *APC* (20), *CCNA* (21), *WT1* (22), *TWIST1* (23), *ISL1* (19), *CDH13* (6, 17), *DCC* (21), *EYA4*, *TAL1*, *SOX1*, *NPY*, and *IPF1* (17) genes. Many other genes proved to be frequently methylated in bladder malignancy. The potential use of these and other tissue biomarkers could also be reproduced in the urine of patients with the disease (9, 23).

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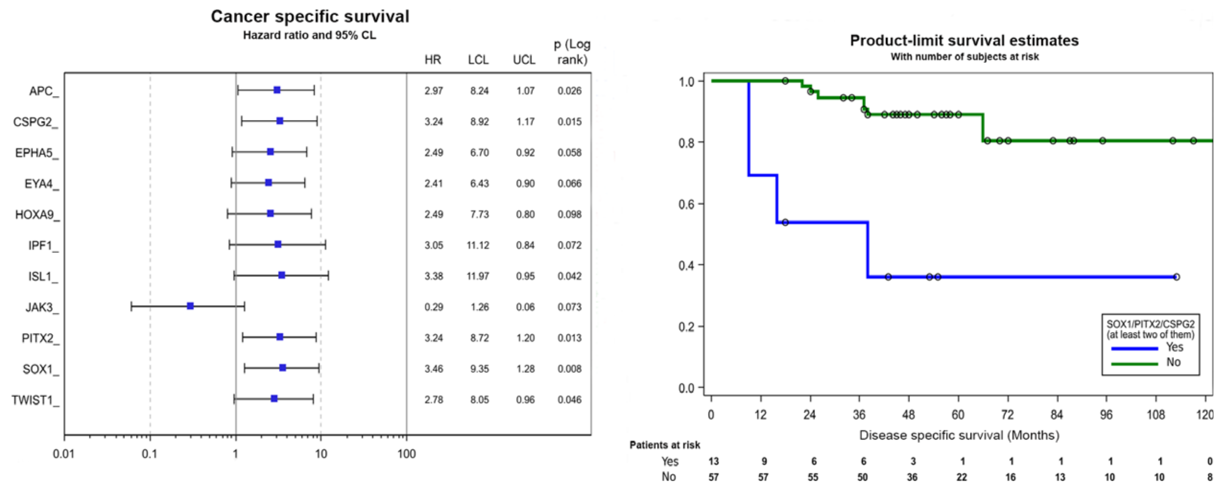

Fig. 3. (A) Hazard Ratio intervals for hypermethylated genes that appear related to disease-specific survival in the population analyzed. *JAK3* hypermethylation implies reduced risk of death of disease while hypermethylation of the rest imply increased risk. (B) Kaplan–Meier curves for cancer-specific survival according to combined hypermethylation of at least two of the three genes (SOX1, PITX2, or CSPG2) compared to only one or none hypermethylated (log-rank, $p < 0.0001$).

Identification of panels of hypermethylated genes could be paramount to develop clinical tests for bladder cancer screening and evaluation of prognosis. By comparing the gene hypermethylation profile of bladder neoplasia we identified panels of genes more frequently hypermethylated in samples from patients with specific clinico-pathological features and also genes which methylation state was related with CSS.

Our data showed for the first time that *JAK3* hypermethylation associated with good prognosis, and the explanation of this fact could be related to changes in epithelial-to-mesenchymal transition (EMT), necessary not only to initiate but also to maintain the metastatic process. Since JAK3 had been related to this process (24) its expression could associate with advanced stages of bladder cancer.

Epithelial-to-mesenchymal transition implies reactivation of an embryonic developmental program that is characterized by loss of E-cadherin (CDH1) expression via the upregulation of genes encoding CDH1-repressing transcription factors, including *TWIST1*, *SMAI1*, and *ZEB1*. Cytoplasmic p27 promotes epithelial–mesenchymal transition and tumor metastasis via STAT3-mediated Twist1 upregulation. A possible mechanism would be PI3K-deregulated p27 binding Janus kinases, to drive STAT3 activation and EMT through STAT3-mediated *TWIST* induction (24).

On the other hand, in our patients, *SOX1*, *PITX2*, and *CSPG2* hypermethylation signaled higher risk of death from bladder cancer. SOX genes (SRY-related high mobility group (HMG) box) encode a family of transcription factors, SOX family members, important mediators of tumorigenesis that may act as oncogenes, tumor suppressor genes, or both depending on the cellular context, and can be activated or inactivated through a variety of genetic and epigenetic mechanisms (25). In most malignant tumors SOX proteins repress Wnt transcriptional responses while Wnt signaling regulates SOX expression resulting in feedback regulatory loops (26). *SOX1* hypermethylation was differentially detected in bladder neoplasia when compared to normal bladder and also tumor adjacent normal tissue and this finding was validated both in tissue and urine sediment (17). Despite its potential use as diagnostic marker, no previous study evaluated this epigenetic change as a prognostic factor.

PITX2 and *GATA6* are characteristically expressed in bladder mesenchymal cells (27). *PITX2* methylation in breast cancer patients has been associated with poor prognosis in several clinical situations (28) and also with biochemical recurrence after radical prostatectomy for prostate neoplasia (29). Conversely in lung cancer patients increased DNA methylation levels of *PITX2* were associated with prolonged survival (28) thus needing further elucidation.

We describe for the first time hypermethylation of *CSPG2* as a marker of prognosis in bladder cancer but accumulated knowledge favors the association between low expression of *CSPG2* and metastatic disease in patients with bladder malignancy by altering inflammation in the tumor microenvironment. RhoGTP dissociation inhibitor 2 (RhoGDI2) suppresses invasion and metastasis in

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52

human bladder cancer cell lines and the proteoglycan versican (chondroitin sulfate proteoglycan 2), a structural component of the extracellular matrix, might mediate part of the GDI2 invasion and metastasis suppressor phenotype (30).

Clearly more studies including larger samples should validate our preliminary data that simultaneous combined hypermethylation of *SOX1*, *PITX2*, or *CSPG2* anticipates mortality in patients with bladder cancer, and if validated this epigenetic profile could help us focus the therapeutic efforts needed to improve survival results in our patients.

CONCLUSIONS

Formerly unreported epigenetic silencing of *HOXA11*, *PENK*, and *CYP1B1* was detected in a Spanish population of patients with bladder neoplasia and that could be a common event with potential use as molecular marker of disease. In addition, hypermethylation of *SOX1*, *PITX2*, and *CSPG2* could discriminate bladder cancer at higher risk of death. On the other hand, hypermethylation of *JAK3* appeared as a marker of favorable prognosis. Additional prospective studies that include larger number of samples are required but this study identified a new hypermethylation profile with important diagnostic and prognostic implications in bladder neoplasia.

CONFLICTS OF INTEREST AND FINANCIAL DISCLOSURES

All authors are devoid of any conflicts of interest associated with the eventual publication of the article. JC Angulo has received educational grants for research from Astellas and Pfizer, and is also a lecturer for Astellas, GSK, and Pfizer; JI López, Ana Martín, M Sánchez-Chapado, A González-Corpas, B Colás, and S Ropero have nothing to disclose.

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