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Prognostic value of opioid binding protein/cell adhesion molecule-like promoter methylation in bladder carcinoma

Sara Duarte-Pereira [a,c], *Filipa Paiva* [a,c], *Vera Lúcia Costa* [a,c], *João Ramalho-Carvalho* [a,c], *Joana Savva-Bordalo* [a,d], *Ângelo Rodrigues* [e], *Franclim Ricardo Ribeiro* [b,c], *Vitor M. Silva* [f], *Jorge Oliveira* [f], *Rui Henrique* [a,e,g], *Carmen Jerónimo* [a,c,g,*]

[a] *Cancer Epigenetics Group, Research Center of the Portuguese Oncology Institute – Porto, Portugal*
[b] *Cancer Genetics Group, Research Center of the Portuguese Oncology Institute – Porto, Portugal*
[c] *Department of Genetics, Portuguese Oncology Institute – Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*
[d] *Department of Medical Oncology, Portuguese Oncology Institute – Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*
[e] *Department of Pathology, Portuguese Oncology Institute – Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*
[f] *Department of Urology, Portuguese Oncology Institute – Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*
[g] *Department of Pathology and Molecular Immunology, ICBAS – Institute of Biomedical Sciences Abel Salazar, University of Porto, Largo Prof. Abel Salazar 2, 4099-003 Porto, Portugal*

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ABSTRACT

The *OPCML* gene (opioid binding protein/cell adhesion molecule-like), a putative tumour suppressor gene, is frequently inactivated in carcinomas, namely through aberrant promoter methylation. Herein, we aimed to determine whether *OPCML* altered expression mediated by epigenetic mechanisms was implicated in bladder carcinogenesis and to assess its potential as a bladder cancer epi-marker.

OPCML promoter methylation levels from 91 samples of bladder urothelial carcinoma, 25 normal bladder tissues and bladder cancer cell lines were assessed by quantitative methylation-specific polymerase chain reaction, and correlated with *OPCML* mRNA expression, determined by quantitative reverse-transcription polymerase chain reaction. To prove the epigenetic regulation of *OPCML*, five bladder cancer cell lines were exposed to 5-aza-2′deoxycytidine (5-aza-dC), a specific DNA methyltransferase inhibitor and trichostatin A (TSA), a histone deacetylase inhibitor.

In bladder tumours, the overall frequency of methylation was 60% and methylation levels were significantly higher when compared with normal mucosa ($P = 0.0001$). No correlation was found between methylation levels and clinicopathological parameters. Interestingly, *OPCML* promoter methylation was associated with worse disease-specific survival ($P = 0.022$) in univariate analysis. Furthermore, a significant inverse correlation between *OPCML* promoter methylation and mRNA expression levels was found, although a significant re-expression was only achieved when 5-aza-dC and TSA were used simultaneously.

The high frequency of *OPCML* promoter methylation in urothelial carcinomas suggests an important role for this epigenetic alteration in bladder carcinogenesis, highlighting its potential as an epigenetic biomarker for bladder urothelial carcinoma with prognostic significance.

* *Corresponding author:* Address: Research Center & Department of Genetics, Portuguese Oncology Institute – Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal. Tel.: +351 961906711; fax: +351 225084016.
E-mail addresses: carmenjeronimo@ipoporto.min-saude.pt, cljeronimo@icbas.up.pt (C. Jerónimo).
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1. Introduction

Bladder cancer is the 11th most common cancer worldwide,[1] accounting for 386,365 new cases and 150,165 deaths per year.[1] In Europe, bladder cancer corresponds to approximately 4.3% of all cancer types.[1] The adjusted incidence and mortality of this disease are approximately three times higher in males than in females. Indeed, male gender, ageing and heavy smoking habits comprise the three main risk factors for bladder cancer development.[1,2]

More than 90% of bladder cancers are urothelial carcinomas and approximately 60% are low-grade superficial papillary carcinomas. The majority of these patients develops cancer recurrence after endoscopic resection, 16–25% of which are high-grade cancers. Only 10% of patients with superficial bladder cancers subsequently develop invasive or metastatic disease. However, almost 25% of patients with newly diagnosed bladder cancer have high histologic grade, muscle-invasive tumours, 50% of which with occult distant metastases.[3]

It is now recognised that many different factors, including genetic and epigenetic alterations, are involved in bladder carcinogenesis and progression. In fact, genetic alterations of specific molecular pathways seem to occur in particular forms of urothelial carcinomas. The genesis of low-grade papillary tumours is linked to constitutively active receptor tyrosine kinase-Ras pathway, whereas high-grade Ta tumours are often characterised by homozygous deletion of the p16/INK4a gene. Conversely, flat carcinoma *in situ* (CIS) and primarily invasive tumours show frequent genetic alterations in the TP53 and RB tumour suppressor genes and respective pathways.[2] Furthermore, several tumour suppressor genes involved in bladder carcinogenesis contain CpG islands at their promoters, making them good targets for epigenetic inactivation. Indeed, in a recent review, Enokida and Nakagawa reported that more than 50 genes, involved in a number of cellular pathways, undergo aberrant promoter methylation in bladder cancer.[4] Besides their biological role in carcinogenesis, these epigenetic alterations may serve as specific tumour markers (epi-markers) as they have been detected not only in tumour tissue samples, but also in serum, bladder washes and urine from bladder cancer patients.[5]

Recent findings demonstrated that the *OPCML* gene (opioid binding protein/cell adhesion molecule-like), also known as *OBCAM* (opioid binding cell adhesion molecule) is frequently inactivated in carcinomas by CpG island methylation, thus constituting an excellent candidate for tumour suppressor gene role, since it is also prone to be inactivated by LOH. Loss of *OPCML* reduces the intercellular adhesion and heterodimeric complex formation, impairing, as well, the subsequent signalling pathways, thereby promoting cancer development.[6] The *OPCML* gene, mapped at 11q25, contains 7 exons codifying for two alternative splice transcript variants (variant 1 and variant 2). The two variants differ in promotor region and firsts exons, encoding two different mature proteins. Sequence analysis of the *OPCML* promoter revealed a GC-rich region spanning 600 bp approximate 500 bp upstream of the translational start site only in the variant 1. Contrarily, variant 2 lacks a CpG island at the promoter region and its expression is mainly restricted to foetal tissues, being barely or even not expressed in adult tissues.[7]

This gene was first identified as a tumour suppressor in epithelial ovarian cancer,[8,9] and later studies refer epigenetic silencing of *OPCML* expression in several other neoplasms, including lung adenocarcinoma,[10,11] colorectal carcinoma[12] and brain cancer.[13] In a recent study, Cui and co-workers evaluated the epigenetic inactivation of the *OPCML* gene in a series of common human tumours, including nasopharyngeal carcinoma, oesophageal, lung, gastric, hepatocellular, colorectal, breast, cervical and prostate carcinomas, as well as lymphomas.[7] Although DNA methylation in bladder cancer has been the object of a significant number of recent reports,[14–20] and some reviews,[4,5,21–23] the *OPCML* promoter methylation has not been investigated, thus far, in bladder cancer.

Therefore, we sought to characterise *OPCML* epigenetic regulation in bladder cancer to determine its potential role in bladder carcinogenesis and whether it might serve as a valuable epi-marker. Specifically, the aims of this study were to (1) assess the promoter methylation status of *OPCML* in primary bladder carcinomas and normal bladder mucosa; (2) correlate the molecular findings with the clinical and histopathological data; and (3) investigate whether epigenetic mechanisms effectively regulate *OPCML* gene expression.

2. Materials and methods

2.1. Patients and samples

The 91 bladder tumour (BT) samples selected for this study correspond to a series of patients diagnosed and treated between 1997 and 2006 at the Portuguese Oncology Institute – Porto, Portugal. Tissue was collected after transurethral resection or radical cystectomy. A small tumour sample was immediately snap-frozen, stored at −80 °C and subsequently cut for DNA extraction after morphological confirmation. The bulk material was routinely fixed in buffered formalin and paraffin-embedded. The corresponding haematoxylin–eosin-stained sections were examined by a pathologist to determine tumour type and grade [according to the ISUP/WHO scheme[24]] and pathological stage (according to the TNM staging system[25,26]). In addition, from nine of the cystectomy specimens, a sample of morphologically normal mucosa adjacent to the tumour (MABT) was in the same way collected and processed. Finally, an independent set of 25 normal bladder mucosa (NBM) samples were used as control. These were obtained from patients submitted to radical prostatectomy for clinically localised prostate cancer. All these patients were classified as pT2 or pT3, based on the pathological findings of the prostatectomy specimen, and thus no case displayed bladder invasion. Relevant clinical data were collected from patient's clinical records. All patients were enrolled after informed consent. These studies were approved by the institutional review board.

2.2. Cell culture and treatment

Bladder cancer cell lines 5637, J82, T24, TCCSUP and SCaBER [ATCC – American Type Culture Collection, MD, USA] were

kindly provided by Prof. Ragnhild A. Lothe, Department of Cancer Prevention, Institute for Cancer Research, Oslo University Hospital, Norway. Except for SCaBER, which derives from a squamous cell carcinoma, cell lines were of urothelial origin. Cells were seeded in 75-cm^3 tissue culture flasks in the correspondent growth medium. To evaluate the epigenetic regulation of the gene, cell lines were exposed to epigenetic-modulating drugs: 5-aza-2′deoxycytidine (5-aza-dC), a specific DNA methyltransferase inhibitor and trichostatin A (TSA), a histone deacetylase inhibitor.

Once cell lines achieved a confluence of 25%, 15 ml of medium were replaced and 5-aza-dC [Sigma, MO, USA] was added in concentrations of 1 μM and 5 μM. In another flask 1 μM of 5-aza-dC and 0.5 μM of TSA [Sigma, MO, USA] were added. One more flask, in which the cells were not exposed to 5-aza-dC or TSA, was used as control (MOCK). The medium and the drugs were changed every 24 h. After 72 h, cells were harvested by tripsinisation and stored at −80 °C. Aliquots for DNA extraction and for RNA extraction were made separately.

2.3. Nucleic acid extraction and bisulphite treatment

DNA from bladder tissues and cell lines was extracted by the phenol–chloroform method, at pH 8, as described by Pearson and Stirling[27] RNA from cell lines was extracted using the RNeasy Mini Kit [QIAGEN, EU], according to the manufacturer's instructions.

All DNA samples were submitted to sodium bisulphite modification, based on the method previously described.[28,29] Briefly, 2 μg of genomic DNA from each sample were used for the chemical treatment. Bisulphite-modified DNA was purified using a vacuum manifold and a Wizard DNA Clean-up System [Promega Corp., WI, USA], treated again with sodium hydroxide, precipitated with ethanol, eluted in 120 μl of water and stored at −80 °C.

2.4. Real-time quantitative MSP (QMSP)

The primers selected for real-time quantitative MSP (QMSP) assays (Genbank no. NM_002545) were designed for a nucleotide sequence located in the promoter region of the *OPCML* gene variant 1 and were optimised for the SYBR Green assay. Forward and reverse oligonucleotides sequences for methylation were, respectively: 5′ GCG CGG TGC GGG TTT ATT TTC 3′ (position −1018 to −997 from the transcriptional start site according to the NCBI database), 5′ TCC CGA TAC CGC CTC GAA ACG AAC G 3′ (position −907 to −882). QMSP reactions were performed in the Applied Biosystems 7500 Real-time PCR system [Applied Biosystems, CA, USA] as follows: each well included 2 μl of modified DNA, 0.5 μl of 10 μM primers (forward and reverse), 10 μl of Power SYBR Green Master Mix [Applied Biosystems] and 7 μl of DEPC treated water (deionised, nuclease-free water). In each of the 96-well plates, multiple negative controls (DEPC treated water) were included, as well as serial dilutions of fully methylated, bisulphite converted DNA – CpGenome Universal Methylated DNA [Millipore, CA, USA] – which were used to construct the standard curve in order to quantify the amount of fully methylated alleles in each reaction.

Running conditions were: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. After 40 cycles, a dissociation-curve analysis was performed using the following conditions: 95 °C for 15 s, 60 °C for 20 s and 95 °C for 30 s. All samples were run in triplicate. The β-actin (*ACTB*) gene was used as an internal reference for normalisation of ratios. The primer sequences were: forward 5′ TGG TGA TGG AGG AGG TTT AGT AAG T 3′ and reverse 5′ AAC CAA TAA AAC CTA CTC CTC CCT TAA 3′, which amplify a 133 bp amplicon. The values obtained, after QMSP analysis (mean quantity) for the *OPCML* gene were divided by the respective value of the internal reference gene. The ratio calculated was then multiplied by 1000 for easier tabulation (methylation level = target gene/reference gene × 1000). This ratio represents an index of input copies of methylated DNA at the primer binding sites, in each sample.

2.5. Quantitative reverse-transcription PCR

Total RNA extracted from cell lines was first transcribed into cDNA using the Revert Aid H Minus First Strand cDNA synthesis kit [Fermentas, EU]. Primers for expression assays were selected to amplify *OPCML* gene variant 1 (Genbank no. NM_002545) and were specifically designed in the transcription region: forward 5′ CGG AGT CCT GGG AAG TTG T 3′ and reverse 5′ TGT CCA TAG CTT TGG GGA AG 3′. The hypoxanthine phosphoribosyltransferase 1 gene (*HPRT1*, Genbank no. NM_000194) was used as internal reference. *HPRT1* primers were 5′ TGA CAC TGG CAA AAC AAT GCA GAC TT 3′ and 5′ TTC GTG GGG TCC TTT TCA CCA GCA A 3′, forward and reverse, respectively.

Quantitative reverse-transcription (QRT)-PCR assays were performed in all cell lines using fluorogenic SYBR Green assays. The reactions were carried out in 96-well plates on an Applied Biosystems 7500 Real-time PCR system using the following conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, and 64 °C (*OPCML/HPRT1*) for 1 min. Each reaction included 2 μl of cDNA, 0.5 μl of 10 μM primers (forward and reverse), 10 μl of Power SYBR Green Master Mix and 7 μl of DEPC treated water. Multiple negative controls were included in each plate. All samples were run in triplicate. The standard curve was constructed with a series of cDNA dilutions obtained from a previously reported positive control (normal colon[7,12]).

2.6. Statistical analysis

The frequency of methylated cases, as well as the median and interquartile range of *OPCML* promoter methylation levels in each group of samples was determined. To categorise samples as methylated or unmethylated for further analysis, a cut-off value was chosen based on the 95 percentile of the methylation levels in NBM samples. Differences in methylation values among the three groups of samples were assessed by the Kruskall–Wallis test, followed by pairwise comparisons (BT versus NBM; MABT versus NBM and BT versus MABT) using the Mann–Whitney *U*-test, when applicable. The Wilcoxon Signed Ranks test allowed the comparison between tumour and corresponding adjacent mucosa methylation levels. Methylation frequencies were compared using the

Chi-square test. The relationship between methylation levels and standard clinicopathological variables [gender, tumour stage and grade, and tumour presentation (primary or recurrence)] were evaluated using the Mann–Whitney or Kruskall–Wallis tests depending on the categorisation of the variables. A Spearman non-parametric correlation test was additionally performed to compare age and methylation levels.

Disease-specific survival curves (Kaplan–Meier with log rank test) were computed for standard variables, such as tumour stage and grade and also for categorised methylation status. A Cox-regression model comprising all significant variables (multivariate test) was computed to assess the relative contribution of each variable to the follow-up status. In multivariate testing, pathological stage was recoded into two groups (Ta–T1 versus T2–4). The statistical value of significance was set at $p < 0.05$. All statistical tests were two-sided. Statistical analysis was performed in the software SPSS version 15.0.

3. Results

3.1. Clinical and pathological data

We tested tissue samples of bladder carcinoma (BT, $n = 91$), mucosa adjacent to bladder tumour (MABT, $n = 9$) and mucosa from normal bladder (NBM, $n = 25$). Seventy-two tumour samples corresponded to primary tumours, whereas the remaining 19 were tumour recurrences. All patients were Caucasian. Relevant clinical and histopathological characteristics of the patients are summarised in Table 1. The median age of the individuals with bladder cancer was significantly higher than that of controls ($p = 0.006$). Follow-up data were available for 67 patients. Whereas 57 patients were submitted to one or multiple transurethral resection procedures, cystectomy was performed in 12 patients. Four of the latter received neoadjuvant chemotherapy and only one received adjuvant chemotherapy.

Table 1 – Demographic characteristics and histopathological data.

	NBM $n = 25$	BT $n = 91$
Gender		
Male	25 (100%)	71 (78%)
Female	0 (0%)	20 (22%)
Age		
Median (range)	65 (48–75)	71 (33–94)
Serum PSA levels		
Median (range)	7.77 (17.35–1.82)	NA
Pathological stage		
Bladder cancer		
pTis	NA	1 (1%)
pTa	NA	31 (34%)
pT1	NA	32 (35%)
pT2	NA	22 (24%)
pT3–4	NA	5 (5%)
Prostate cancer		
pT2	11 (44%)	NA
pT3	14 (56%)	NA
Histopathological classification		
Papillary, low-grade	NA	30 (33%)
Papillary, high-grade	NA	39 (43%)
Invasive, high-grade	NA	21 (23%)
Carcinoma *in situ*	NA	1 (1%)

Abbreviations: NBM, normal bladder mucosa (from prostate cancer patients); BT, bladder tumour; and NA, not applicable.

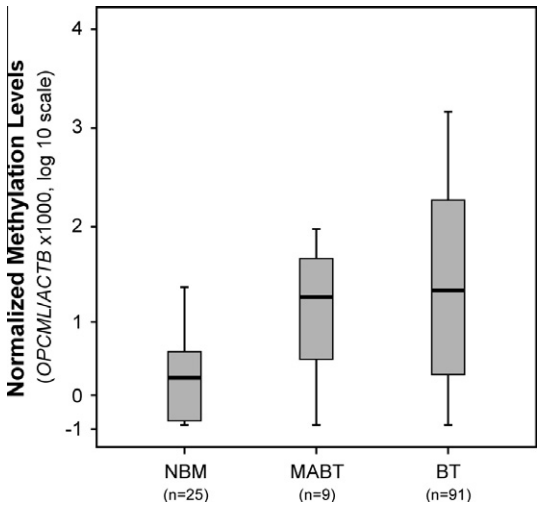

Fig. 1 – Distribution of *OPCML* promoter methylation levels in bladder tissues. Box-plot of *OPCML* promoter methylation levels in normal bladder mucosa (NBM), mucosa adjacent to bladder tumour (MABT) and bladder tumours (BT).

3.2. Methylation analysis of bladder tissues

The distribution of *OPCML* promoter methylation levels and frequencies among BT, MABT and NBM are displayed in Fig. 1 and Table 2. The frequency of promoter methylation was defined based on a cut-off value of 11.5, as described in Section 2. *OPCML* methylation frequency was significantly lower in NBM compared to either MABT or BT ($p < 0.001$ in both cases) but no difference was found between the latter. The Kruskall–Wallis test detected significant differences in methylation levels among the three groups of samples

Table 2 – Frequency and distribution of *OPCML* promoter methylation in bladder tissues.

	NBM $n = 25$	MABT $n = 9$	BT $n = 91$
Methylation levels			
Median (range)	2.0 (0.0–23.8)	18.7 (0.0–94.7)	22.0 (0.0–1465.2)
Methylation frequencies			
n (%)	1 (4.0)	6 (66.7)	54 (59.3)

Abbreviations: NBM, normal bladder mucosa; MABT, mucosa adjacent to bladder tumour; and BT, bladder tumour. Threshold for methylation status was defined at 11.5 based on the 95 percentile value of NBM samples.

Table 3 – Association of clinicopathological parameters and *OPCML* promoter methylation in 91 bladder tumours.

	Frequency (%)	*p*-value[a]	Levels median (range)	*p*-value[b]
Gender		0.440		0.229
Male	62.0		10.9 (0.0–1465.2)	
Female	50.0		18.4 (0.0–719.7)	
Age		0.254		0.101
<65	50.0		12.4 (0.0–691.1)	
⩾65	63.5		52.2 (0.0–1465.2)	
Tumour presentation		0.438		0.270
Primary	56.9		22.0 (0.0–1465.2)	
Recurrence	68.4		36.7 (0.1–691.0)	
Histopathological classification		0.159		0.146
Papillary, low-grade	50.0		8.6 (0.0–822.3)	
Papillary, high-grade	56.4		22.0 (0.0–1456.2)	
Invasive, high-grade	76.2		129.7 (0.0–691.1)	
Carcinoma *in situ*	100.0		22.0	
Pathological stage		0.205		0.247
pTis	100.0		22.0	
pTa	48.4		10.5 (0.1–730.6)	
pT1	56.3		17.9 (0.0–1465.2)	
pT2	77.3		122.2 (0.0–394.7)	
pT3–4	60.0		342.6 (0.1–691.1)	

[a] Chi-square or Fisher's test, based on the categorisation of the variables.
[b] Non-parametric Kruskal–Wallis or Mann–Whitney tests, based on the categorisation of the variables.

($p = 0.0001$). Pair-wise comparisons showed that MABT and BT methylation levels were statistically similar and significantly higher than those of NBM ($p = 0.009$ and $p = 0.0001$, respectively).

3.3. *Associations between methylation and clinicopathological data*

Analyses between clinicopathological parameters and molecular data are summarised in Table 3. No statistically significant difference in *OPCML* methylation levels between males and females was apparent. Although a statistically significant difference was not found when BT cases were age-categorised (<65 or ⩾65 years), a weak correlation between age and methylation levels was found within BT patients when a Spearman correlation analysis was carried out ($r_s = 0.262$; $p = 0.012$). Given the age differential between NBM and BT groups, a possible influence of age on the statistical results was assessed. An analysis comprising only age-matched BT and NBM cases ($n = 45$) was thus performed. In this subgroup of samples, *OPCML* methylation levels remained significantly higher in BT than in NBM samples ($p = 0.001$), indicating that methylation differences were not age-related.

The distribution of methylation levels along each histological category is displayed in Fig. 2. Although a trend for increased promoter methylation levels in high-grade invasive carcinomas is observed, the Kruskall–Wallis test did not reveal significant differences. Moreover, no differences in methylation levels were found between primary and recurrent tumours, nor among the different pathological stages.

The median follow-up of the 67 patients with available information was 60 months (range: 1–120 months). At the time of the last follow-up, 45 patients were alive with no evidence of cancer and 2 patients were alive with cancer progression, whereas 20 patients were deceased, 12 of which due to bladder cancer. Disease-specific survival analysis showed that patients with unmethylated *OPCML* promoter had a significantly better prognosis ($p = 0.022$, Fig. 3a). In univariate analysis, both histopathological type and pathological stage showed prognostic information ($p = 0.0001$ for both variables, Fig. 3b and c), but not age or gender. In multivariate analysis using pathological stage, histopathological classification and

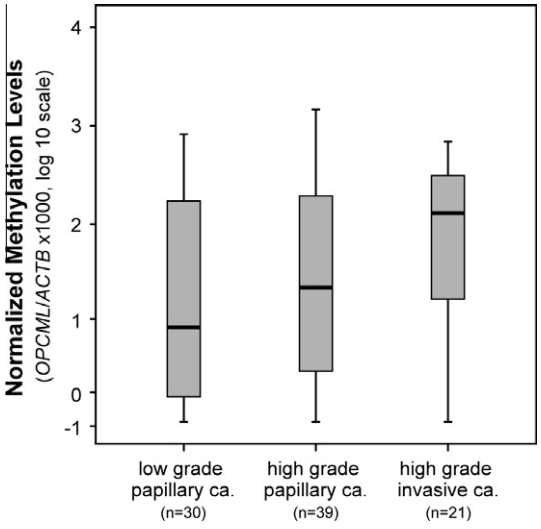

Fig. 2 – *OPCML* promoter methylation levels in 91 bladder tumours. Box-plot of *OPCML* promoter methylation levels across tumour subgroups. The single carcinoma *in situ* in this series is not depicted.

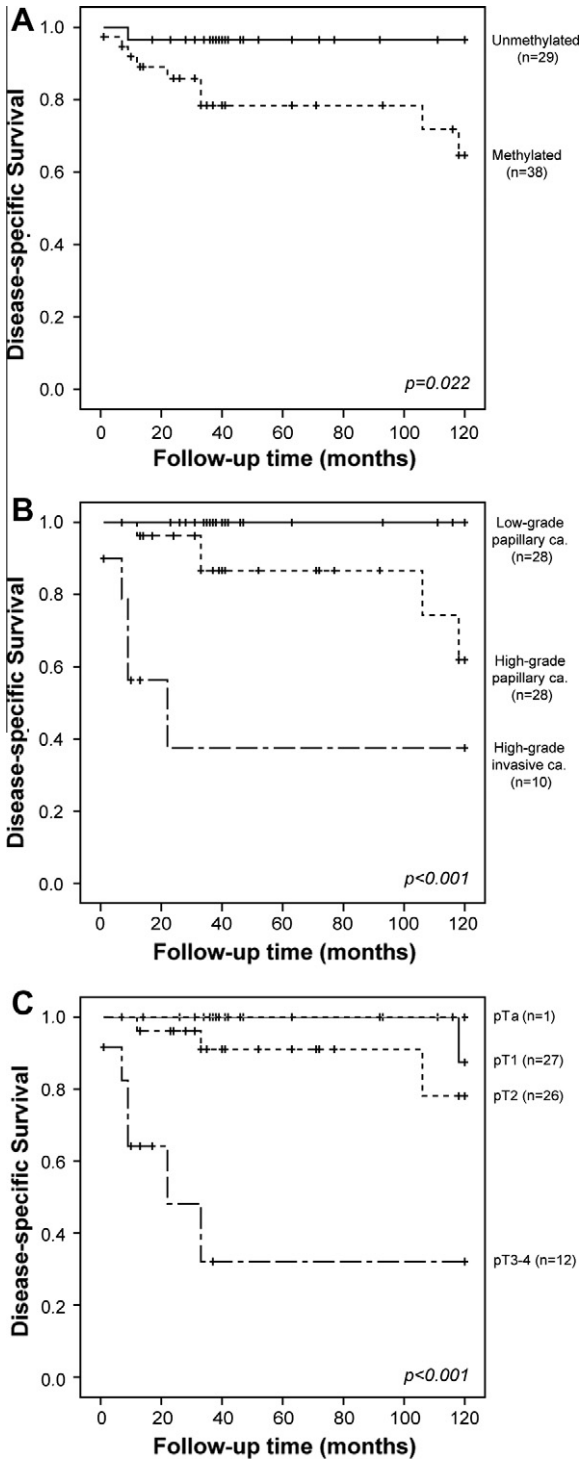

Fig. 3 – Disease-specific survival according to molecular and pathological parameters. (A) *OPCML* promoter methylation status; (B) histopathological classification; and (C) pathological stage.

OPCML methylation status, a high pathological stage (pT2-4, hazard ratio (HR) = 14.4, 95% confidence interval (CI) 3.4–60.8, $p < 0.001$) and *OPCML* methylation (HR–11.1, 95% CI 1.3–98.0, $p = 0.030$) were selected in the final model as independent predictors of disease-specific survival.

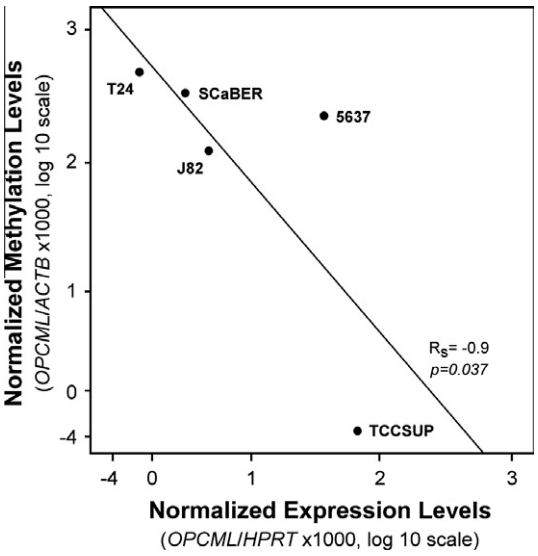

Fig. 4 – Correlation between *OPCML* promoter methylation and mRNA expression levels in bladder cancer cell lines. Methylation levels were calculated as *OPCML/ACTB* × 1000, whereas expression levels were calculated as *OPCML/HPRT* × 1000.

Table 4 – *OPCML* promoter methylation and mRNA expression levels in five bladder cancer cell lines.

Cell line	Methylation levels[a]	Expression levels[b]
5637	225.7	37.8
J82	123.1	4.3
T24	479.8	0.6
TCCSUP	0.0	68.2
SCaBER	334.9	2.5

[a] Levels calculated as *OPCML/ACTB* × 1000.
[b] Levels calculated as *OPCML/HPRT* × 1000.

3.4. *Methylation and expression analysis of cell lines*

To assess whether *OPCML* is epigenetically regulated in bladder cancer, five bladder carcinoma cell lines were exposed to epigenetic-modulating drugs. In mock cell lines, a strong inverse correlation was found between *OPCML* methylation and expression levels ($r_s = -0.9$, $p = 0.037$, Fig. 4 and Table 4). Epigenetic-modulating treatment results are depicted in Fig. 5. Although some variation among cells lines was apparent, methylation levels tended to decrease following exposure to increased concentrations of 5-aza-dC. Overall, re-expression levels of *OPCML* mRNA were higher when 5-aza-dC and TSA were used in combination.

4. Discussion

The *OPCML* gene, which encodes a cell adhesion molecule, has been recently reported as a tumour suppressor gene in several common human malignancies, including ovarian cancer,[8,9] lung adenocarcinoma,[10,11] brain tumours,[13] gastro-

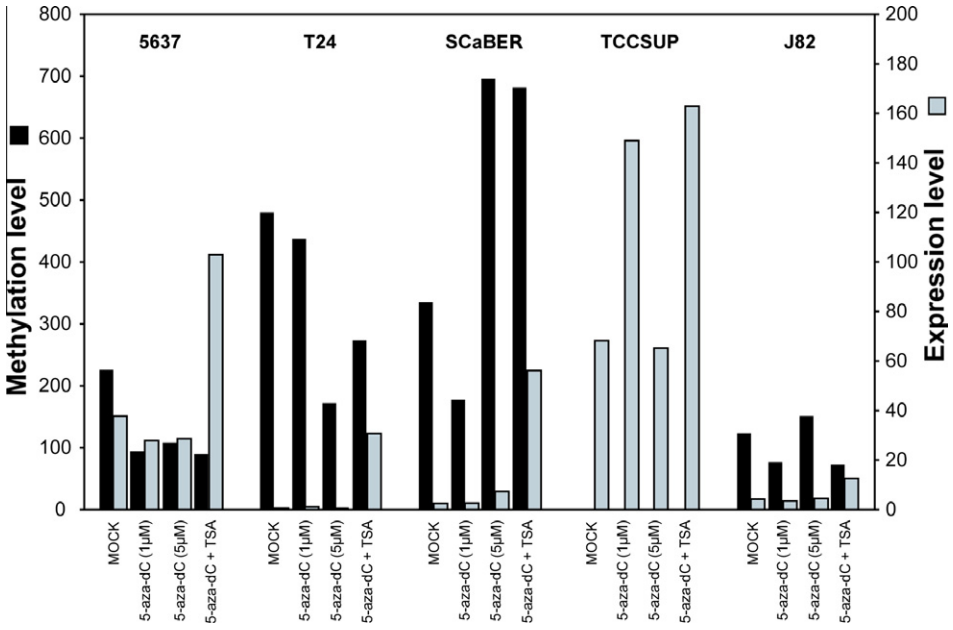

Fig. 5 – Effect of epigenetic-modulating drugs in *OPCML* promoter methylation and mRNA expression levels in bladder cancer cell lines. Methylation levels were calculated as *OPCML/ACTB* × 1000, whereas expression levels were calculated as *OPCML/HPRT* × 1000. Abbreviations: 5-aza-dC, 5-aza-2′deoxycytidine and TSA, trichostatin A.

intestinal, breast and prostate cancer.[7] Because a CpG island was found near the transcription start site, an epigenetic-mediated regulation of gene expression was postulated.[7] Owing to the reported tumour-specificity of *OPCML* promoter methylation,[7] it might constitute a valuable tumour marker. We thus sought to characterise *OPCML* epigenetic regulation in bladder cancer.

In this study, *OPCML* promoter methylation was detected in most bladder carcinomas, irrespective of stage and histopathological type. Thus, it is suggested that this is an early and common alteration across the different pathways already established for bladder carcinogenesis.[2] This finding is further supported by the detection of significant *OPCML* methylation levels in morphologically normal urothelium adjacent to bladder cancer, which might be explained by the well-known field effect or field carcinogenesis typical of bladder cancer.[30,31] Alternatively, this observation might be explained by age-related *OPCML* promoter methylation. However, the lack of correlation between age and *OPCML* promoter methylation levels in NBM samples argues against that hypothesis. Eventually, this epigenetic alteration might identify urothelial cells that although not displaying recognisable morphological features of malignancy have an already neoplastic genotype. Indeed, the tumour-specificity of *OPCML* promoter methylation was also confirmed, as only 4% of normal bladder epithelium samples tested positive and the respective methylation levels were significantly lower than in bladder tumours. Thus, *OPCML* promoter methylation seems a promising marker for bladder cancer detection in urine, although its diagnostic coverage is only about 60%, according to our results, thus requiring the addition of other epi-markers for that purpose. However, because prostate[7] and renal (C. Jeronimo, unpublished observations) cancers are seldom methylated at the *OPCML* promoter, this biomarker might be particularly useful for differential detection of urological malignancies in urine samples.[21]

Interestingly, a significant, albeit weak, correlation between methylation levels and ageing was detected in bladder cancer samples. Whereas age-related methylation is a known phenomenon that could account for these finding,[32,33] our results show that *OPCML* promoter methylation is indeed tumour specific, because the significantly higher methylation levels were retained when cancer patients were age-matched with normal tissue providers. Moreover, no correlation was found between *OPCML* methylation levels and other clinicopathological parameters, nor between primary and recurrent tumours, emphasising the potential value of *OPCML* promoter methylation as an early and specific bladder cancer biomarker.

Remarkably, our results also sustain that *OPCML* hypermethylation status may carry prognostic value in bladder cancer. Patients with tumours unmethylated at the *OPCML* promoter showed a significantly better disease-specific survival in both univariate and multivariate analyses comprising clinicopathological variables. Pathological stage also emerged as a significant predictor of outcome in these analyses, as patients with invasive tumours (pathological stage T2–4) showed a much worse outcome. This epigenetic alteration may thus be useful in the distinction between bladder tumours with similar morphology and clinicopathological characteristics, but with a different biological and clinical behaviour, even if this hypothesis requires validation in a larger series of patients. From a biological standpoint, epigenetic silencing of *OPCML* is likely to impair intercellular adhesion and cell signalling, thus promoting tumour progression. Hence, the acquisition of this epigenetic alteration should provide a selective growth advantage to cancer cells and might account for the more clinically aggressive behaviour. Indeed, the functional role of *OPCML* as a tumour suppressor gene was clearly established in previous

reports, although in different tumour models. Using a monolayer colony formation assay and the soft agar assay, Cui and co-workers[7] demonstrated that ectopic expression of *OPCML* significantly inhibited the anchorage-dependent growth and reduced the colony formation efficiency, in three different cancer (colon, prostate and oesophageal) cell lines. Moreover, Sellar et al.[9] verified that *OPCML* re-expression resulted in a higher rate of cell aggregation while reducing cell growth in an ovarian cancer cell line and documented a significant inhibition of tumour growth in subcutaneous and intraperitoneal models in nude mice.[9] Hence, there is solid evidence of the tumour suppressor function of *OPCML* across diverse tumour models which, in similarity with bladder cancer, display *OPCML* silencing through promoter methylation.

Since epigenetic regulation of the *OPCML* gene has been documented previously in other tumour models,[7,9,34] we exposed bladder cancer cell lines to epigenetic-modulating agents. In our mock cancer cell lines a significant inverse correlation between *OPCML* promoter methylation and corresponding mRNA expression levels was found, which is consistent with a methylation-related silencing of gene expression. To further confirm these observations, methylation and expression levels were determined in cell lines exposed to different concentrations of a demethylating agent, 5-aza-dC and in combination with TSA, a HDAC inhibitor. We found that re-expression of *OPCML* mRNA was maximal when a combined treatment was performed. Thus, besides aberrant DNA methylation, histone acetylation seems to play a role in *OPCML* regulation. In addition, when cell lines were exposed to 5-aza-dC alone, only a weak increase in mRNA expression was apparent, notwithstanding the decrease in promoter methylation levels. Because histone modifications and CpG methylation are functionally linked, it was reasonable to expect that epigenetic regulation of *OPCML* gene would depend on both mechanisms. A more dominant role for histone modifications seems plausible, but definite answers require further studies to assess mRNA re-expression levels following exposure to TSA alone.

Summarising, our results indicate that *OPCML* is epigenetically down-regulated in bladder cancer, and that this event carries prognostic significance for these patients. The high frequency of promoter methylation found in tumours and adjacent morphologically normal urothelium, contrasting with infrequent and low level methylation found in normal mucosa, further suggests that this is a specific cancer-related event that occurs early in bladder carcinogenesis. Moreover, owing to the scarcity of this epigenetic alteration in prostate and renal cancer, it represents a promising epi-marker for early and accurate detection of bladder cancer.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.12.025.

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