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Original Paper

Discovery of myopodin methylation in bladder cancer

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Abstract

Myopodin is an actin-binding protein that shuttles between the nucleus and the cytoplasm. After identifying an enriched CpG island encompassing the transcription site of myopodin, we aimed at evaluating the potential relevance of myopodin methylation in bladder cancer. The epigenetic silencing of myopodin by hypermethylation was tested in bladder cancer cells ($n = 12$) before and after azacytidine treatment. Myopodin hypermethylation was associated with gene expression, being increased *in vitro* by this demethylating agent. The methylation status of myopodin promoter was then evaluated by methylation-specific polymerase chain reaction (MS-PCR) analyses. Myopodin was revealed to be frequently methylated in a large series of 466 bladder tumours (68.7%). Myopodin methylation was significantly associated with tumour stage ($p < 0.0005$) and tumour grade ($p = 0.037$). Myopodin expression patterns were analysed by immunohistochemistry on tissue arrays containing bladder tumours for which myopodin methylation was assessed ($n = 177$). The presence of low nuclear myopodin expression alone ($p = 0.031$) or combined with myopodin methylation ($p = 0.008$) was associated with poor survival. Moreover, myopodin methylation in 164 urinary specimens distinguished patients with bladder cancer from controls with a sensitivity of 65.0%, a specificity of 79.8%, and a global accuracy of 75.3%. Thus, myopodin was identified to be epigenetically modified in bladder cancer. The association of myopodin methylation and nuclear expression patterns with cancer progression and clinical outcome, together with its ability to detect bladder cancer patients using urinary specimens, suggests the utility of incorporating myopodin methylation assessment in the clinical management of patients affected by uroepithelial neoplasias.

Keywords: **bladder cancer; myopodin; methylation**

Introduction

Bladder cancer can be described as a genetic disease, driven by the multistep accumulation of genetic and epigenetic factors [1,2]. The most common epigenetic event in the human genome is the addition of methyl groups to the carbon-5 position of cytosine nucleotides [3]. CpG islands are present in half of human genes and typically overlap with promoters and first exons of genes [3–7]. Transcriptional inactivation of cancer-related genes by CpG island promoter hypermethylation has also been shown in bladder cancer [8–12].

Myopodin, also known as synaptopodin 2, is a dual compartment protein that displays actin-bundling activity and redistributes between the nucleus and the cytoplasm in a differentiation-dependent and stress-induced manner [13,14]. Deletions of the SYNPO2 gene at 4q26 and losses of protein expression of myopodin have been reported to be associated with aggressive prostate cancer using *in vitro, in vivo*, and clinical material [15–18]. In bladder cancer, previous results showed differential nuclear localization of myopodin among bladder cancer cells while sharing structural cytoplasmic expression patterns [19]. In bladder tumours, the loss of nuclear myopodin was associated with increased tumour staging and poor clinical outcome [19]. The impact of myopodin in nuclear transport has also been described in non-neoplastic cells [20–23], including myocytes [22]. In an attempt to uncover the mechanisms by which

myopodin is lost in bladder progression, we tested the hypothesis of epigenetic silencing, being aware that other mechanisms including deletions or mutations could be involved in such a process and that further studies are warranted to evaluate such hypotheses in depth. An enriched 5′-CpG island was identified around the transcription start site of myopodin, supporting its susceptibility to be epigenetically modified by hypermethylation. To the best of our knowledge, myopodin had not been reported to be epigenetically altered in bladder cancer or any other tumour type. Here the impact and clinical relevance of myopodin methylation in bladder cancer progression were evaluated using *in vitro* strategies, as well as on tissue and urinary specimens. Our results showed that myopodin was hypermethylated and is potentially useful as a tumour stratification biomarker and a clinical outcome prognosticator for patients with bladder cancer.

Materials and methods

Tumour samples

Primary bladder tumours were collected following the guidelines for the protection of human subjects at seven participating hospitals. The initial series included 46 frozen bladder tumours for which we had previously performed gene expression profiling analyses using oligonucleotide arrays [24]. From these specimens, paired normal urothelium was available in a subset of cystectomized invasive bladder tumours ($n = 20$). These samples served (a) to screen the myopodin methylation rate; (b) to test the association of myopodin expression in bladder cancer progression; and (c) to test the association of myopodin transcript expression with its methylation status. This set included non-muscle invasive lesions ($n = 9$): pTa ($n = 2$), pT1 ($n = 7$); and muscle invasive tumours ($n = 37$): pT2 ($n = 6$), pT3 ($n = 28$), pT4 ($n = 3$). The distribution of the tumour grade of these non-muscle and muscle invasive tumours together was low grade ($n = 4$) and high grade ($n = 42$), both defined under standard criteria [25]. An independent set of 466 paraffin-embedded bladder tumours served (a) to validate myopodin methylation rates; (b) to assess the feasibility of myopodin methylation analyses in paraffin-embedded material; (c) to test the association of myopodin protein expression with its methylation status; and (d) to evaluate the association of myopodin methylation and protein expression with clinicopathological variables. This set comprised non-muscle invasive lesions ($n = 285$): pTa ($n = 44$), pT1 ($n = 233$) pTis ($n = 8$); and muscle invasive tumours ($n = 181$): pT2 ($n = 110$), pT3 ($n = 50$), pT4 ($n = 21$). The distribution of the tumour grade of these non-muscle and muscle invasive tumours together was low grade ($n = 104$) and high grade ($n = 362$). OCT and paraffin-embedded bladder tumours were macrodissected based on haematoxylin and eosin evaluations to ensure a minimum of 75% of tumour cells [24].

Methylation analyses of the promoter of myopodin

A specific search for enrichment of CpG in the promoter of myopodin (Figure 1A) was performed using CpG Island Searcher software, publically available at http://www.cpgislands.com. The methylation status of myopodin was analysed by two polymerase chain reaction (PCR) analysis strategies of bisulphite-modified genomic DNA, which induces the chemical conversion of unmethylated, but not methylated, cytosine to uracil. First, genomic sequencing of both strands of myopodin promoter was analysed after bisulphite treatment of genomic DNA. A second strategy used PCR with primers specific for either the methylated or the modified unmethylated DNA (MS-PCR) [12,26]. Primer sequences for bisulphite sequencing and unmethylated and methylated reactions were designed encompassing its transcription start site distant at 322 base pairs before its ATG starting codon (Figure 1B and Table 1). Genomic DNA was extracted using standard methods. DNA from normal lymphocytes treated *in vitro* (IVD) with SssI methyltransferase was used as a positive control for methylated alleles. DNA from normal lymphocytes was used as a positive control for unmethylated alleles. PCR products were loaded onto non-denaturing 2% agarose gels, stained with ethidium bromide, and visualized under an ultraviolet transilluminator [12,26].

RNA and protein analysis of myopodin in bladder cancer cell lines

Human bladder cancer cell lines ($n = 12$) [27] were treated with 1 μM and 5 μM 5-aza-2′-deoxycytidine (AZA; Sigma) for 72 h to achieve demethylation [12,26]. RNA was isolated using standard methods [27]. RNA (1 μg) was reverse-transcribed using AMV Reverse Transcriptase (Promega) and amplified

Table 1. Primer sequences and PCR conditions for bisulphite sequencing (SEQ), methylated (MSP) and unmethylated specific PCR (USP), and RT-PCR for myopodin

	Sense primer (5′ → 3′)	Antisense primer (5′ → 3′)	Product size (bp)	Annealing temperature (PCR cycles)
SEQ	TTTTTTGTGTGTGAAGTTGTTTT	CCCCTATACCCATATTTTCCTT	322	60 (40)
MSP	TTTTTTCGAAGGTGGGTC	CAACCGCCTACTACGAATTT	125	55 (37)
USP	TTTTTTTTTGAAGGTGGGTT	CCAACCACCTACTACAAATTTA	129	55 (37)
RT-PCR	CATCAAGCTCATGGAAAGCA	TTCTGTGCACTGGGTCTTTG	183	60 (28)

using specific primers and conditions for myopodin (Table 1). PCR was performed using a final volume of 15 μl containing 1× PCR Ecostart buffer (Ecogen), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.25 μM of each primer, and 1.5 U of Ecostart *Taq* polymerase (Ecogen). For PCR amplification, 0.4 μg of cDNA was utilized. RT-PCR primers were designed between different exons and encompassing large introns to avoid any non-specific amplification of genomic DNA. GADPH was used as an internal control to ensure cDNA quality and loading accuracy. The amplification product was resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining [12,26].

Immunofluorescence

Cells were grown on coverslips in P6 dishes, fixed in 4% formaldehyde, and fluorescently stained [12,26]. In order to monitor AZA exposure, cells were stained for myopodin at 1/500 dilution for 45 min, using an anti-myopodin antibody (rabbit polyclonal; kindly provided by Dr Mundel, previously tested in refs 13 and 19). The secondary antibody was used at 1/250 dilution. Confocal optical sections were obtained using a Leica TCS SP microscope equipped with krypton and argon lasers. Images were acquired and processed using Leica LCS Lite software.

Tissue microarrays

We constructed four different bladder cancer tissue microarrays including a total of 177 bladder tumours among the patients recruited for assessing myopodin methylation. Three cores of each of the tumours under study were included in the corresponding tissue arrays. The tumour stage distribution was pT1 (81), pT2 (50), pT3 (28), and pT4 (18). The tumour grade distribution was low grade (9) and high grade (168). Their clinicopathological information and annotated follow-up in a subset of 93 bladder tumours allowed the evaluation of associations of myopodin methylation and nuclear protein expression patterns among them and with clinicopathological variables.

Immunohistochemistry

Protein expression patterns of myopodin were assessed at the micro-anatomical level by immunohistochemistry on these tissue microarrays using the antibody mentioned above at 1/250 dilution following standard avidin–biotin immunoperoxidase procedures, using the antibody and conditions previously reported [19]. The biotinylated secondary antibody (Vector Laboratories) was used at 1 : 1000 dilution.

Urinary samples

Urine specimens of individuals presenting microscopic haematuria under suspicion of bladder cancer were collected immediately before urinary cytology and/or cystoscopy. Samples were handled anonymously following ethical and legal guidelines at three participating hospitals. The presence of bladder cancer was confirmed by cystoscopy, the gold standard [25]. Genomic urinary DNA was extracted using standard methods, and 250 ng was treated with bisulphite as shown above [12,26]. Urinary specimens ($n = 164$) served to analyse the clinical utility of myopodin methylation at discriminating patients with bladder cancer ($n = 40$) from controls ($n = 124$), including healthy individuals ($n = 54$) and patients with benign urological diseases such as urinary tract infections ($n = 32$), benign prostatic hyperplasia ($n = 28$), and kidney stones ($n = 10$), all of them confirmed to be negative for bladder cancer by urinary cytology and cystoscopy. Histopathological information after subsequent surgical interventions provided the tumour stage distribution among positive cases for bladder cancer: pTa (12), pTis (2), pT1 (11), and pT2+ (15). The tumour grade distribution was low grade (19) and high grade (21).

Statistical analysis

Associations among myopodin methylation and protein expression patterns with tumour stage and grade were evaluated using non-parametric Wilcoxon–Mann–Whitney and Kruskall–Wallis tests [28]. Associations of methylation and protein expression patterns with overall survival were evaluated in those cases for which follow-up information was available, using the log-rank test [28]. Overall survival time was defined as the years elapsed between surgery and death from disease (or the last follow-up date). Patients who were alive at the last follow-up or lost were censored. Survival curves were plotted using Kaplan–Meier methodology. Associations among protein patterns and methylation of myopodin were analysed using Kendall's tau test. The diagnostic performance of myopodin methylation measured by MS-PCR in urinary specimens was evaluated through the estimation of its sensitivity, specificity, and diagnostic accuracy. Sensitivity was defined by the number of urine specimens methylated for myopodin among cases with confirmed bladder cancer by the gold standard. Specificity was defined by the number of urine specimens unmethylated for myopodin among urine samples belonging to controls without bladder cancer as confirmed by cystoscopy and urinary cytology findings. Accuracy was defined by the number of correctly classified cases and controls based on myopodin methylation patterns among all the cases under study together [28].

Results

Myopodin CpG island hypermethylation in bladder cancer cell lines

An enriched CpG island was found to be located around the transcription start site of myopodin

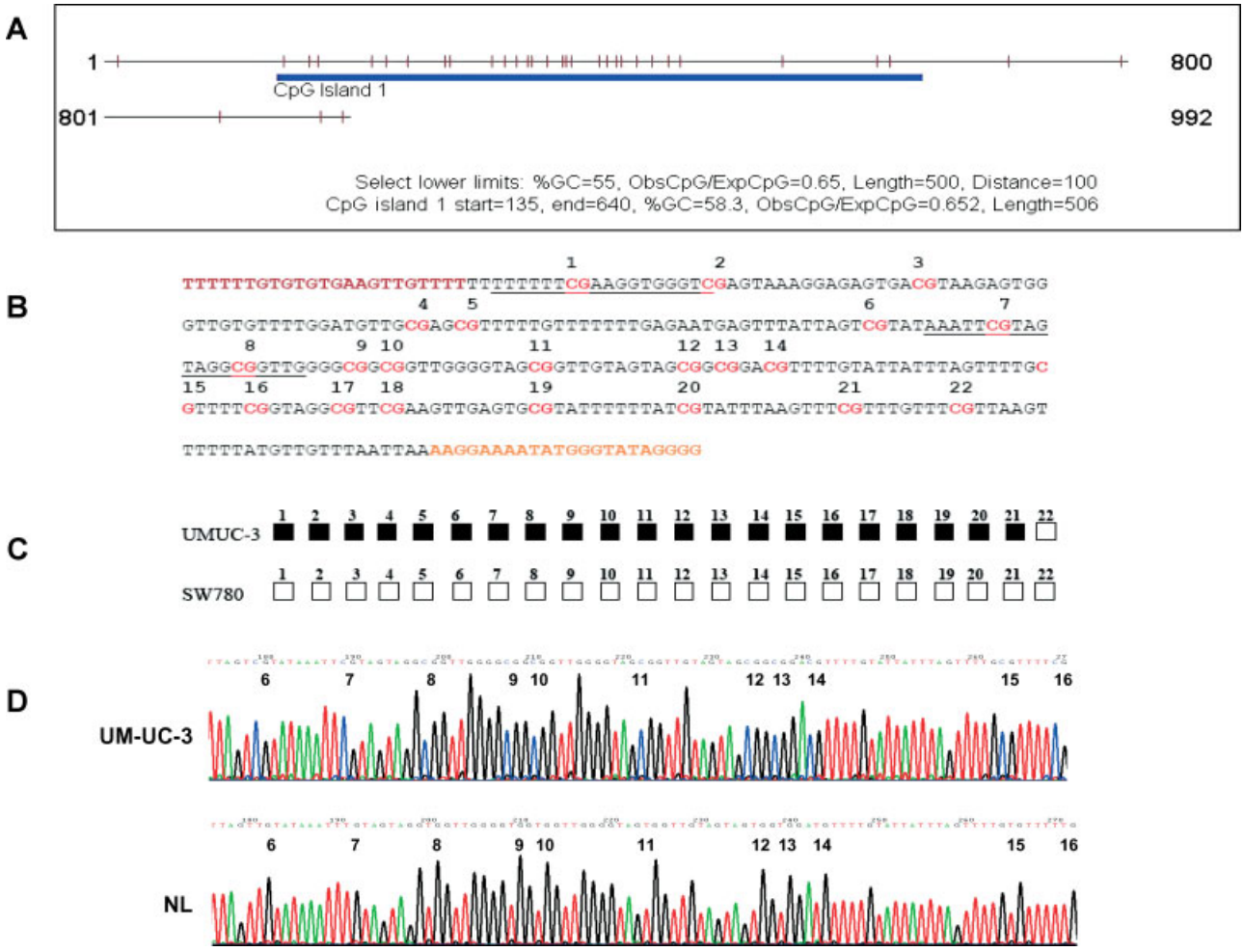


Figure 1. Identification of myopodin methylation in bladder cancer cell lines. (A) Identification of an enriched CpG island encompassing the transcription site of myopodin. Output of the specific search for enrichment of CpG in the promoter of myopodin performed using CpG Island Searcher software. (B) Schematic depiction of the myopodin CpG islands around its transcription start site. Nucleotide sequences of the CpG island region analysed by bisulphite sequencing; the sequencing primers are highlighted in yellow and red, and the primers utilized for MS-PCR are underlined. (C) Analysis of CpG island methylation status of the promoter of myopodin by bisulphite genomic sequencing in bladder human cancer cell lines. CpG dinucleotides are represented in squares. The presence of 'Cs' in the dinucleotide CpG reflects methylated cytosines (black squares), while the presence of 'Ts' in the dinucleotide CpG reflects unmethylated cytosines (white squares). Black squares indicate the presence of methylation confirmed in at least two of the clones that were sequenced for the cell lines under analysis. (D) Chromatograms obtained by bisulphite genomic sequencing in bladder human cancer cell lines. Representative examples of the sequences obtained in UM-UC-3 and SW780, methylated and unmethylated bladder cancer cell lines, respectively. Normal lymphocytes (NL) were also found unmethylated

(Figure 1A), supporting its susceptibility to be epigenetically modified. Myopodin was initially tested as a candidate gene for hypermethylation in bladder cancer cells. In order to assess myopodin methylation status, 12 human bladder cancer cell lines were initially screened using bisulphite genomic sequencing and methylation-specific PCR (MS-PCR) targeted to the areas surrounding its transcription start site (Figure 1B). Bisulphite sequencing of these cell lines revealed the CpG island methylation for UM-UC-3 (Figures 1C and 1D). Among the normal tissues analysed, normal urothelium and lymphocytes (NL) were found unmethylated at the myopodin CpG island promoter. Unmethylated bladder cancer cell lines served as negative sequencing controls as well, as shown, for example, for SW780 (Figures 1C and 1D).

Screening of 12 human bladder cancer cell lines using MS-PCR revealed CpG island myopodin methylation for UM-UC-3 and EJ138 (Figure 2A). The link between hypermethylation and gene silencing was established by the treatment of methylated and unmethylated bladder cancer cell lines with a DNA demethylating agent. Exposure of the methylated bladder cancer cell lines EJ138 and UM-UC-3 to the demethylating drug, 5-aza-2′-deoxycytidine (AZA), increased the expression of myopodin at the transcript level (Figure 2B). SW780 was used as a control cell line to assess the specificity of AZA exposure not to modify myopodin expression in unmethylated bladder cancer cells (Figure 2B). Immunofluorescence analyses were performed in order to confirm that myopodin protein expression was also

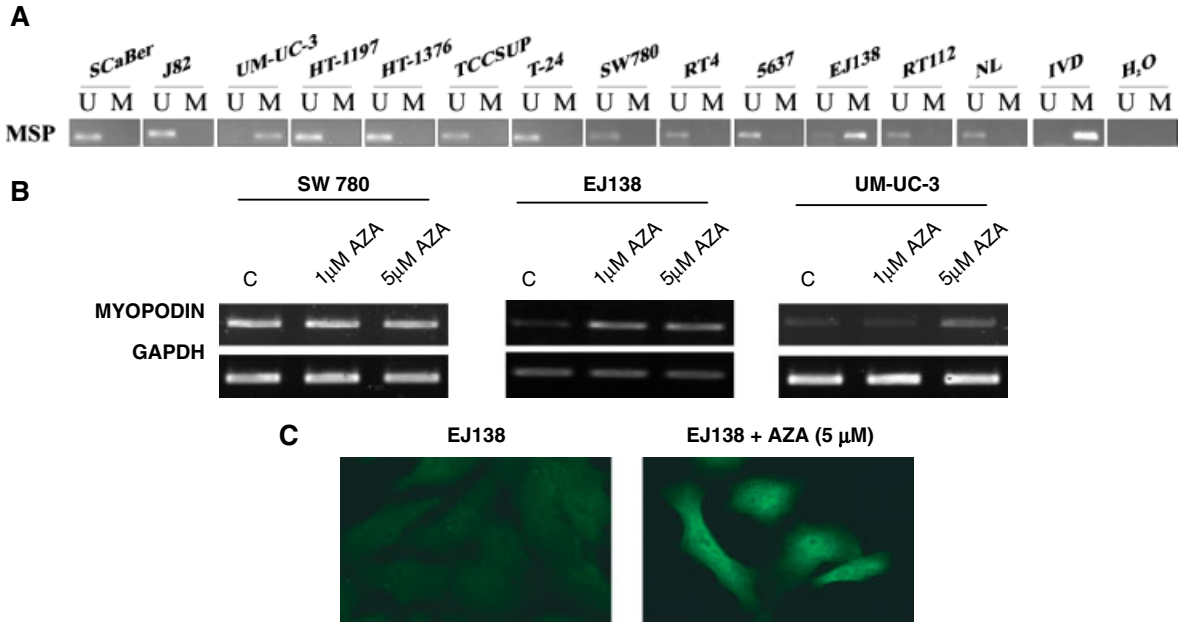


Figure 2. CpG island methylation was associated with myopodin silencing. (A) Methylation-specific PCRs (MSP) for myopodin in human bladder cancer cell lines. The presence of a PCR band in lane M indicates a methylated myopodin, while the presence of a PCR band in the U indicates an unmethylated gene. Normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) were used as negative and positive controls for unmethylated and methylated myopodin, respectively. (B) Treatment with the demethylating agent AZA reactivated the transcript expression of myopodin. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of myopodin expression. GAPDH expression is shown as transcript loading control. Myopodin transcript expression increased in the methylated cell lines EJ138 and UM-UC-3 after AZA exposure. The unmethylated cell line (SW780) did not show changes in myopodin expression. (C) Treatment with the demethylating agent AZA increased the protein expression of myopodin. Immunofluorescence analysis of myopodin expression after AZA exposure, which reactivated the protein expression in the methylated cell line EJ138

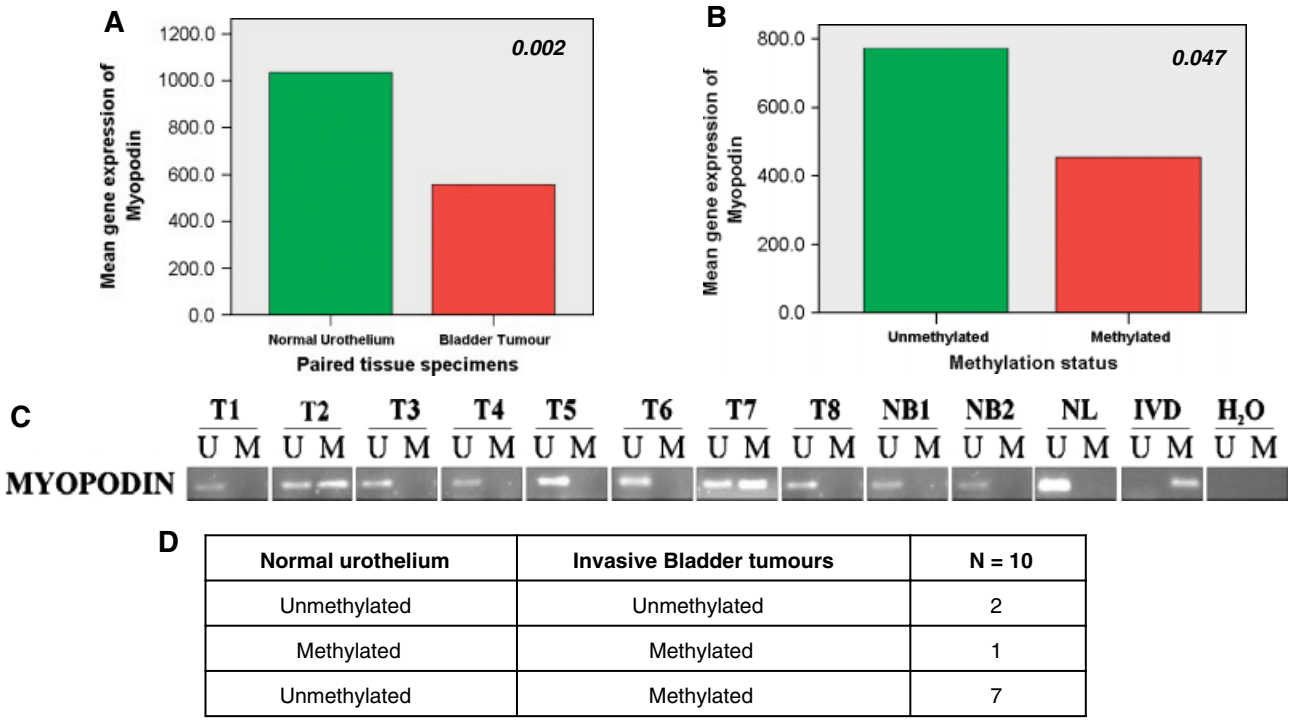
increased after AZA exposure, as shown for EJ138 (Figure 2C). Overall, the AZA reactivation analyses indicated a high correlation of increasing gene and protein expression and myopodin methylation status.

Myopodin is frequently hypermethylated in primary bladder tumours and is associated with tumour progression

Once the functional consequences of myopodin CpG island hypermethylation were determined *in vitro*, we evaluated its impact in human bladder tumours. First of all, the methylation status of myopodin was analysed in a set of frozen bladder tumours ($n = 46$) for which we had previously performed gene expression profiling analyses using oligonucletide arrays [24]. Initial screening in this subset of frozen primary bladder tumours revealed that myopodin hypermethylation was present in 86.9% of the cases ($n = 46$). Moreover, the methylation status of myopodin was associated with an increased tumour grade (Mann–Whitney, $p = 0.017$). The differential transcript expression levels of myopodin were compared in a subset of pairs of frozen bladder tumours and normal urothelium ($n = 20$). Interestingly, significantly lower myopodin gene expression was observed in bladder tumours compared with their respective paired normal urothelium (Mann–Whitney, $p = 0.002$) (Figure 3A). Moreover, myopodin transcript expression was found to be significantly associated with its methylation status ($p = 0.047$) (Figure 3B).

These results prompted us to perform further MS-PCR analyses on an independent set of bladder tumours, in this case extracting genomic DNA from paraffin-embedded material (Figure 3C). A high methylation rate for myopodin was observed (68.7%) in this large set of bladder tumours ($n = 466$). It was also tested whether myopodin hypermethylation was cancer-specific. Comparison of the methylation of ten bladder tumours and their respective pairs of normal urothelium obtained in cystectomized invasive patients revealed that myopodin methylation was a frequent event in association with cancer progression (Figure 3D).

The next analyses dealt with evaluating the link between the hypermethylation status of myopodin and clinicopathological variables of bladder cancer. Tumours displaying advanced T2+ tumours were more frequently methylated than those with non-muscle invasive tumours (Table 2A). Increased methylation rates were also observed in high-grade bladder tumours (Table 2B). Interestingly, myopodin methylation was found to be significantly associated with increased tumour stage (Kruskall–Wallis, $p < 0.0005$), and increased tumour grade (Mann–Whitney, $p = 0.037$). Overall, these results indicated that myopodin hypermethylation is a frequent event in bladder cancer in association with pathological indicators of tumour progression.



D

Normal urothelium	Invasive Bladder tumours	N = 10
Unmethylated	Unmethylated	2
Methylated	Methylated	1
Unmethylated	Methylated	7

Figure 3. Myopodin hypermethylation was shown to be a cancer-specific event. (A) Myopodin transcript levels decreased in pairs of bladder tumours. Transcript levels analysed using oligonucleotide arrays were differentially expressed between normal urothelium and bladder tumours ($p = 0.002$). (B) Myopodin transcript levels decreased in association with myopodin methylation status. Transcript levels analysed using oligonucleotide arrays were differentially expressed between methylated and unmethylated bladder tumours ($p = 0.047$). (C) Myopodin methylation was detected in paraffin-embedded pairs of bladder tumours and normal urothelium. Representative MS-PCR analyses for myopodin using primary bladder tumours and normal urothelium. Normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) were used as negative and positive controls for unmethylated and methylated myopodin, respectively. (D) Myopodin methylation increased in bladder tumours compared with their paired normal urothelium. Summary of the methylation status of myopodin analysed by MS-PCR in pairs of primary bladder tumours and normal urothelium

Myopodin protein expression patterns correlated with methylation, tumour progression, and clinical outcome of patients with bladder tumours

The next set of analyses dealt with the association of the promoter methylation of myopodin with its protein expression patterns. The goal was to link the novel findings of myopodin methylation with the silencing of the gene and the coded product. To address this issue, the protein expression patterns of myopodin were evaluated by immunohistochemistry in several tissue arrays containing bladder tumours for which the myopodin methylation status was assessed. Protein expression patterns of myopodin were observed in the membrane, cytoplasm, and nucleus. In this subset of tumours ($n = 177$), myopodin methylation was also found to be significantly associated with tumour stage (Kruskall–Wallis, $p = 0.0005$) and tumour grade (Mann–Whitney, $p = 0.024$). The cytoplasmic myopodin expression remained unchanged with regard to the clinicopathological tumour stage, grade, and myopodin methylation status. Interestingly, the loss of nuclear expression of myopodin was also found to be associated with increased tumour stage (Kruskall–Wallis, $p = 0.036$) and tumour grade (Mann–Whitney, $p = 0.004$), as previously reported [19]. A significant association was found between myopodin methylation status and nuclear expression lower than the cut-off of 10% (Kendall's tau $= 0.678$, $p < 0.0005$). It was observed that the presence of nuclear expression lower than 10% in combination with myopodin methylation was also associated with tumour stage ($p = 0.036$). In a subset of pT2+ bladder tumours with available follow-up ($n = 93$), having a nuclear protein expression of myopodin lower than 10% was significantly associated with a shorter overall survival (log-rank, $p = 0.031$, Figure 4A). Myopodin methylation itself was nearly associated with poorer survival (log rank, $p = 0.06$). More interestingly, the combined presence of nuclear expression below 10% and myopodin methylation was strongly associated with poorer survival (log-rank, $p = 0.008$, Figure 4B). Overall, these analyses revealed that the loss of nuclear protein expression was associated with methylation, increased tumour stage, and poor overall survival. Thus, the combination of these variables (myopodin methylation and nuclear expression) could be considered likely prognosticators of tumour progression and poor outcome in patients with bladder cancer.

Myopodin hypermethylation in urinary specimens distinguished bladder cancer patients from controls

Once the biological and clinical relevance of myopodin hypermethylation was evaluated in bladder cancer cell

Table 2. Summary of the distribution of the promoter CpG island methylation status of myopodin obtained by MS-PCR regarding tumour stage and grade. (A) Myopodin methylation was associated with increasing tumour stage ($p < 0.0005$). (B) Myopodin methylation was associated with increasing tumour grade ($p = 0.037$)

(A) Stage	N = 466	Percentage
pTa	44	9.4%
Methylated	20	45.5%
Unmethylated	24	54.5%
pT1	233	50.0%
Methylated	147	63.1%
Unmethylated	86	36.9%
pTis	8	1.7%
Methylated	2	25.0%
Unmethylated	6	75.0%
pT2	110	23.6%
Methylated	92	83.6%
Unmethylated	18	16.4%
pT3	50	10.7%
Methylated	43	86.0%
Unmethylated	7	14.0%
pT4	21	4.5%
Methylated	16	76.2%
Unmethylated	5	23.8%

(B) Grade	N = 466	Percentage
Low grade	104	22.3%
Methylated	65	62.5%
Unmethylated	39	37.5%
High grade	362	77.7%
Methylated	255	70.4%
Unmethylated	107	29.6%

lines and tumours in association with clinicopathological variables, we then tested whether myopodin methylation could be detected in urinary specimens by means of MS-PCR (Figure 4C). Myopodin methylation in the 164 urinary specimens distinguished patients with bladder cancer ($n = 40$) from controls ($n = 124$) with a sensitivity of 65.0% (25/40), a specificity of 79.8% (99/124), and a global accuracy of 75.3% (125/166). Overall, these results indicated that the methylation of myopodin was detectable in the urine. Moreover, they supported a diagnostic role for myopodin methylation in distinguishing bladder cancer patients from controls with a high sensitivity and specificity.

Discussion

Our study identified the epigenetic silencing of myopodin and showed the clinical relevance of myopodin methylation in uroepithelial tumours. The consequences of the novel CpG island hypermethylation of myopodin in bladder cancer progression can be assessed from the standpoint of mechanistic and translational implications. Mechanistically, it is important to evaluate the cellular consequences of myopodin promoter methylation. Expression estimates of myopodin were linked to methylation analyses. AZA exposure experiments confirmed the impact of methylation in myopodin expression by specifically restoring myopodin transcript and protein expression in methylated cells. Overall, *in vitro* analyses revealed that myopodin was aberrantly silenced by CpG island promoter hypermethylation. In concordance, transcript levels of myopodin were shown to be significantly lower in methylated bladder tumours. In this line of argument, a statistically significant association was found between methylation and low nuclear protein expression of myopodin by immunohistochemistry on tissue arrays, while the cytoplasmic expression remained unaffected by the myopodin methylation status. Although its role in cancer progression has not been elucidated, the epigenetic silencing of myopodin might aid in understanding how myopodin may contribute to bladder tumourigenesis and tumour progression. Future studies are warranted to dissect such specific mechanisms in the context of bladder cancer and other human neoplasias.

The translational implications of the discovery of myopodin methylation have been strongly addressed in this work. The association of myopodin methylation with bladder cancer progression can be justified as follows. Two different approaches using two independent sets of tumours served to link methylation to cancer. First, comparison of the transcript levels of paired tumours and normal urothelium revealed significantly lower myopodin transcript levels in bladder tumours in association with their methylation status. In addition, the cancer specificity evaluation by MS-PCR in pairs of bladder tumours and normal urothelium related myopodin methylation to bladder cancer progression. Second, it was shown that the methylation of myopodin is a frequent event among bladder tumours using a large series of patients with bladder cancer ($n = 466$), regardless of the source of genomic DNA being from frozen or paraffin-embedded material. Third, myopodin methylation was significantly associated with increasing tumour stage and tumour grade. In addition to the clinicopathological stratification of patients with bladder cancer, a relevant translational point relates to treatment. In this new scenario, myopodin represents a potential therapeutic target whose expression could be potentially reactivated by demethylation drugs.

A further step in the clinical evaluation of myopodin in bladder cancer progression deals with analyses of its nuclear expression patterns by immunohistochemistry on bladder tumours of known methylation status. Immunostaining assessed the associations of the loss of nuclear myopodin protein expression with increasing tumour stage and grade, confirming previous observations [19]. The novelty of this report in this regard is the link of the low nuclear expression with methylation. The analyses of the protein expression patterns of myopodin served not only to associate the novel findings of myopodin methylation with the silencing of the gene and the coded product, but also to confirm the previously reported results regarding the clinical relevance of the nuclear protein expression of

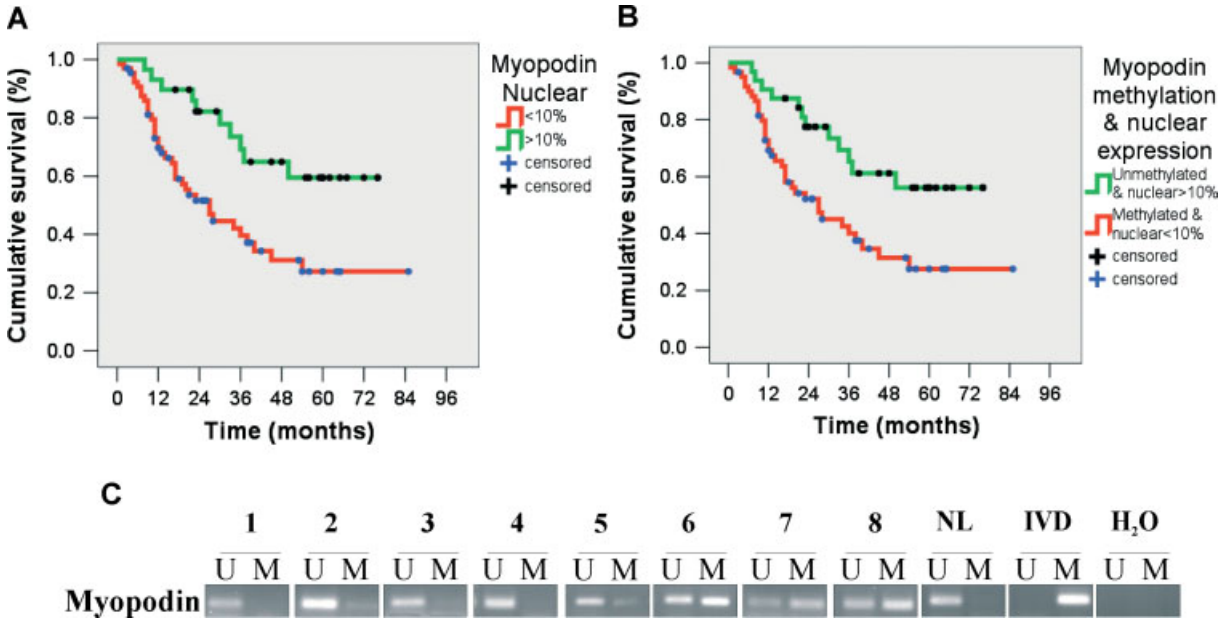


Figure 4. Nuclear protein expression patterns and myopodin methylation are associated with clinical outcome in bladder cancer. (A) Loss of nuclear myopodin expression alone was associated with overall survival. Kaplan–Meier curve survival analysis indicated that nuclear protein expression of myopodin lower than 10% measured by immunohistochemistry on tissue arrays was associated with poor survival (log rank, $p = 0.031$). (B) Loss of nuclear myopodin protein expression in combination with myopodin methylation was associated with overall survival. Kaplan–Meier curve survival analysis indicated that tumours with nuclear expression of myopodin lower than 10% and methylated for myopodin had a poorer survival (log rank, $p = 0.008$). Urinary methylation of myopodin distinguished patients with bladder cancer from controls. (C) Myopodin methylation can be detected in urinary specimens. Representative MS-PCR analyses for myopodin using urinary specimens of controls without bladder cancer (lines 1–4) and patients with bladder tumours (lines 5–8). Normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) were used as negative and positive controls for unmethylated and methylated myopodin, respectively

myopodin in bladder cancer. An additional goal was to assess how methylation could further discriminate clinical outcome in combination with myopodin protein expression patterns. Interestingly, the presence of low nuclear myopodin protein levels alone or in combination with myopodin methylation was associated with poor survival. Myopodin methylation increased the discrimination among patients with good or poor outcome obtained by the protein expression patterns alone, as shown by the p values. Thus, the combination of epigenetic and proteomic analyses has served to discover a novel gene epigenetically modified that was previously reported to be differentially expressed in bladder cancer progression in association with clinicopathological variables and clinical outcome.

A relevant step in the clinical evaluation of myopodin in bladder cancer progression deals with analyses of myopodin methylation patterns in urinary specimens. Interestingly, the measurement of myopodin methylation in these non-invasive specimens served to distinguish patients with bladder cancer from controls with high diagnostic accuracy. The majority of positive cases unmethylated for myopodin had a pTaG1 tumour. Interestingly, myopodin methylation in urinary specimens rendered a higher sensitivity than urinary cytology, a reference criterion in bladder cancer diagnostics [25]. Assessing myopodin methylation in this non-invasive body fluid represents a potential alternative adjunct for the early detection and follow-up of these patients.

In summary, our study identified the novel methylation of myopodin. It provided a mechanistic explanation for the observed loss of myopodin in uroepithelial malignancies by epigenetic silencing. Hypermethylation emerged as a strong indicator of tumour progression for bladder cancer patients. The loss of nuclear myopodin protein expression alone or in combination with myopodin methylation stratified bladder tumours histopathologically and predicted clinical outcome. Finally, myopodin methylation in urinary specimens played a diagnostic role for bladder cancer. These observations support the introduction of myopodin assessment for the stratification and clinical management of patients affected by uroepithelial neoplasias.

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