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Prognostic relevance of methylation markers in patients with non-muscle invasive bladder carcinoma

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Abstract

There is increasing evidence for the role of epigenetic gene silencing in superficial bladder cancer. The aim of the current study was to investigate the prognostic value of epigenetic alterations in patients with non-muscle invasive bladder carcinoma. We checked the methylation status of 20 cancer associated genes (*p14ARF*, *p16 CDKN2A*, *STAT-1*, *SOCS-1*, *DR-3*, *DR-6*, *PIG-7*, *BCL-2*, *H-TERT*, *BAX*, *EDNRB*, *DAPK*, *RASSF-1A*, *FADD*, *TMS-1*, *E-Cadherin*, *ICAM-1*, *TIMP-3*, *MLH-1*, *COX-2*) for DNA methylation. We analysed microdissected tumour samples from 105 consecutive patients with primary non-muscle invasive bladder carcinoma. Quantitative methylation analysis of CpG sites in the promoter region of the genes was performed with methylation sensitive quantitative real time PCR ('Methylight'). Univariate analysis for association with tumour recurrence was carried out with the Kaplan–Meier analysis and the log-rank test. Follow-up data were available in 95/105 patients (91.4%). A tumour recurrence was observed in 26 patients (27.3%). We could identify six genes (*SOCS-1*, *STAT-1*, *BCL-2*, *DAPK*, *TIMP-3*, *E-Cadherin*), where methylation was associated with tumour recurrence. In Kaplan–Meier analysis, *TIMP-3* showed a significant association with recurrence free survival. Methylation of *TIMP-3* predicted prolonged disease free interval. In this study, we report a comprehensive analysis on prognostic relevance of gene methylation in non-muscle invasive bladder cancer. We identified one gene (*TIMP-3*) where methylation was associated with a more favourable outcome. Our data strongly support the usefulness of gene methylation as a prognostic marker in patients with non-muscle invasive bladder cancer.

Keywords: Bladder; Methylation markers; Non-invasive; Cancer associated genes

1. Introduction

More than 50,000 new cases of bladder cancer are diagnosed in the U.S. each year. Approximately 75–85% of the tumours are confined to the mucosa or the submucosa. Although these tumours can be removed by transurethral resection, more than 50% will recur [1]. Depending on various risk factors including invasion of the submucosa, tumour focality and size, the progression rate varies from 7.1% for patients with a low-risk tumour up to 40% for patients with high-risk bladder carcinoma. Depending on initial staging and grading there is a 4.3% risk for patients with low-risk tumours and of up to 30% for patients with high-risk tumours to die from initial non-invasive disease [2].

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There is a clear need to identify patients with high-risk of tumour progression to allow improved bladder cancer management. On the other hand to avoid over treatment, it is also crucial to identify patients with low-risk of tumour recurrence and progression. Since genetic changes are relatively rare in non-muscle invasive bladder cancer compared to invasive tumours, there is increasing interest in epigenetic changes in cancer development. Hypermethylation of promoter regions of tumour suppressor genes is now the most well categorised epigenetic change in human neoplasia [3]. It has become apparent that aberrant promoter methylation can be associated with loss of gene function that can provide a selective advantage to neoplastic cells [4]. Therefore, promoter methylation has been proposed to be a third way in the carcinogenesis according to Knudsons "two hit hypothesis" [5]. Furthermore, methylation has been described as a valuable marker for cancer detection and as a prognostic marker [3]. Interestingly, the usefulness of methylation markers has also been proven to detect possible decrease in gene function [6,3] . In contrast to other tumour entities only few data are available on hypermethylation studies on bladder cancer. Among the studied genes that were found to display hypermetylation in bladder cancer are endothelin receptor b (*EDNRB*) [7], RAS association domain family 1A (*RASSF1A*) [8], death association protein kinase *DAPK* [9], *E-Cadherin* [10–12], and *TPEF* (transmembrane protein containing epidermal growth factor and follistatin domains) [13]. The majority of these reports focus on single genes and on a small cohort of patients. Despite these limitations it has been shown that hypermethylation of *DAPK* is associated with a higher rate of disease recurrence. We have recently described the frequent methylation of apoptosis-related genes in bladder cancer [6].

From a clinical point of view the most promising application for methylation analysis is the detection of cancer or the utilisation of methylation as a prognostic marker.

The aim of the current study is to analyse the prognostic value in the methylation status of the 5′ region of cell cycle regulatory genes (*p14ARF*, *p16CDKN2A*, *STAT-1* [signal transducer and activator of transcription 1], *SOCS-1* [suppressor of cytokine signaling]), apoptosis-related genes (*DR-3*, *DR-6*, *PIG-7*, *BCL-2*, *H-TERT*, *BAX*, *EDNRB*, *DAPK*, *RASSF-1A*, *FADD*, *TMS-1*), cell adhesion and metastasis associated genes (*E-Cadherin*, *ICAM-1* [intercellular cell adhesion molecule-1], *TIMP-3* [tissue inhibitor of metalloproteinase 3]), the mismatch repair gene *MLH-1*, and the angiogenesis-related Cyclooxigenase-2 (*COX-2*) gene. The majority of these genes, including *p14ARF*, *p16CDKN2A*, [14], *SOCS-1* [15], *BCL-2* [16], *H-TERT* [17], *EDNRB* [7], *DAPK* [14,9], *RASSF-1A* [18], *TMS-1* [19], *E-Cadherin* [11], *ICAM-1* [20], *TIMP-3* [21], *MLH-1* [14], have been described to be methylated in several tumour entities. An involvement of *STAT-1* methylation in the regulation of the gene expression was suggested since *STAT-1* expression can be enhanced by inhibition of DNA-methyltransferases [22]. For other of these genes (*DR-3*, *DR-6*, *PIG-7*(*LITAF*), *BAX*, *FADD*) no data are available investigating the methylation status in human cancers.

We studied the methylation status of a cohort of 105 patients with primary non-muscle invasive bladder carcinoma using the 'Methylight' technique, which utilises fluorescence-based real-time PCR (TaqMan) technology. Methylight is a highly sensitive assay, capable of detecting methylated alleles in the presence of a 10,000-fold excess of un-methylated alleles [23].

2. Materials and methods

2.1. Patients

We obtained tumour material from 105 consecutive patients undergoing transurethral resection of a primary tumour at the department of Urology, at the University Hospital Hamburg-Eppendorf. The age of patients ranged between 33 and 86 years with a median age of 68 years. 32 (26%) were female and 90 (73%) were male.

Pathological diagnosis was established using Haematoxilin/Eosin sections and the tumours were classified according to the 5th edition of the UICC and the WHO. Histopathological examination revealed 81 pTa tumours and 24 pT1 tumours, with 23 tumours classified as grade 1; 81 tumours as grade 2; and 1 tumour as grade 3.

Adjuvant therapy: Post-operative adjuvant intravesical therapy after transurethral resection with chemo- or immunotherapy was administered in 91 of 105 patients (86.7%).

Clinical follow-up: Follow-up investigations were performed by office urologists according to the guidelines of the European Association of Urology, and included cystoscopy every 3 months. All tumour recurrences were histologically confirmed.

2.2. Microdissection and DNA-preparation from paraffin embedded specimens

To study methylation patterns in non-muscle invasive bladder cancer, microdissection was performed in samples from 105 patients undergoing transurethral resection for primary bladder carcinoma. Therefore, 1–3 parallel sections were dewaxed, hydrated and briefly stained with hematoxilin/eosin. Tumour tissue was removed from the slides under microscopic control using a sterile needle. The microdissected tissue fragments were digested with 1 mg/ml proteinase K (Sigma–Aldrich, Deisenhofen, Germany) and DNA was extracted using a commercially available kit (Qiamp Viral Mini Kit, Qiagen, Hilden, Germany). Sodium bisulfite treatment and DNA-preparation was performed as described before [6].

2.3. Quantitative methylation sensitive real-time PCR

Methylation analysis was performed with fluorescence based real-time PCR (Opticon, MJ Research,Waltham, MA) similar to the Methylight technique described by Eads *et al*. [23]. Control reactions were carried out for non-CpG sites of 2 control genes, *Actin B* and *Collagen IIa*. Genomic DNA (Genomic DNA male, Promega) that was treated with *SssI* was used as a fully methylated reference. The percentage of fully methylated DNA (PMR value) was calculated as the ratio between a sample and the standards. Each set of primers and probe were designed to amplify a sequence overlapping at least five CpG sites. The primer designs used for PCR are shown in Table 1. PCR conditions consisted of an initial denaturation for 10 min at 91 °C followed by 50 cycles of denaturation for 15 s at 91 °C, following annealing for 1 min at 59 °C. Methylation levels were calculated as PMR (percentage of fully methylated reference). For descriptive evaluation all tumours showing at least a weak positive PCR were estimated as positive (PMR $\geqslant$ 0.1%). Methylation levels were classified as negative (PMR $<$ 0.1%), low (PMR 0.1–3.9%), positive (4.0–19.9%), intermediate (PMR 20.0–49.9%), high (PMR 50.0–74.9%) and extremely high (PMR $\geqslant$ 75%), similar as suggested by Eads *et al*. [23]. Occasionally PMR values may be >100%, in cases when the *SssI* treatment of the standard DNA was not complete or in cases of aneuploidy of the gene locus of interest.

2.4. Statistical analysis

Statistical analysis was performed with commercially available software SAS (SAS Institute Inc., Cary, NC). The Kaplan–Meier method and the log-rank test were used to derive the recurrence-free survival function, and the log-rank test to compare curves for two or more groups. For the Kaplan–Meier analysis the median PMR level was used as a cut-off level. Therefore, the definition varied for each gene with the aim of getting equal sample sizes for each KM curve. This gives the best power to find a difference if there is a linear association. *P*-values of <0.05 were considered to reflect significant differences between groups.

3. Results

3.1. Methylation patterns in bladder tumour samples

3.1.1. Frequency of methylated tumours according to the single genes

Methylation patterns were assessed in 105 tumour samples from primary bladder carcinoma. According to the percentage of methylated tumours, we identified four groups of genes. In the first group, *DR-3*, *ICAM-1*, and *COX-2* were methylated in >70% of the samples. The second group, including *EDNRB*, *RASSF-1A*, *BCL-2* displayed methylation in 40–69% of the tumour samples. The third group, consisting of *SOCS-1*, *STAT-1*, *p16CDKN2A*, *H-TERT*, *DAPK*, *TIMP-3*, and *MLH-1*, showed methylation in 10–39% of the tumours, whereas *p14ARF*, *DR-6*, *TMS-1*, *PIG-7*(LI TAF), *BAX*, *FADD*, and *E-Cadherin* were methylated in none of the tumours, or only in single cases (Table 2a).

3.1.2. Quantitative methylation analysis

Methylation levels for the single genes as a percentage of fully methylated reference (PMR) was quantitatively investigated. As median, very high level of methylation (PMR $\geqslant$ 75%) was detected in *DR-3*. A high level of methylation (PMR 50–74.9%) was detected in *SOCS-1* and *BCL-2*. An intermediate PMR (PMR 20–49.9%) level was detected in *EDNRB*, *RASSF-1A*, and *ICAM-1*. A methylation level of 4–19% was detected in *H-TERT*, *DAPK*, *E-Cadherin*, *COX-2* and *MLH-1*. *STAT-1*, *p16CDKN2A*, *p14ARF*, *DR-6*, *TIMP-3* displayed a faint methylation (PMR 0.1–3.9%). *TMS-1*, *PIG-7*(*LITAF*), *BAX*, *FADD* displayed no methylation (PMR $<$ 0.1%) (Table 2b). Significant differences ($P < 0.05$) in PMR levels between pTa and pT1 tumours were detected in *BCL-2*, *RASSF-1A*, *TIMP-3*, *SOCS-1*. According to histopathologic grading, significantly different PMR levels were found in *BCL-2*, *EDNRB*, *H-TERT*, and *COX-2*.

3.2. Clinical follow-up

Complete follow-up data were available in 95/105 patients (91.4%). 10 patients were lost of follow-up. Among the 95 patients in who follow-up data were available, 26 (27.3%) experienced tumour recurrence. In 22 patients additional information were available concerning histopathologic staging and grading. 16 patients had a pTa recurrence, 5 patients had a pT1 recurrence, and 1 patient had a CiS recurrence.

3.3. Clinical relevance of methylation status

To identify genes, that might be relevant for prediction of tumour recurrence, we first compared the frequencies of methylation in tumours from patients with recurrent and non-recurrent tumours for the single genes. We could identify six genes (*SOCS-1*, *STAT-1*, *BCL-2*, *DAPK*, *TIMP-3*, *E-Cadherin*), that showed a >10% difference in the percentage of methylated tumour samples (Tables 3a and 3b). These genes were *SOCS-1* and *STAT-1* in the group of cell cycle regulatory genes; *BCL-2* and *DAPK* in the group of apoptosis-related genes; and *TIMP-3* and *E-Cadherin* in the cell adhesion and metastasis genes. In *STAT-1*, *TIMP-3*, *E-Cadherin*

Table 1
Design and location of the oligonucleotides used for Methylight

Hugo gene nomenclature	Alternate name	GenBank Accession No	Amplicon location relative to transcription start (bp)	Mean distance from transcription start (bp)	Location of amplicon in gene	%GC	Forward primer sequence	Reverse primer sequence	Probe oligo sequence
ACTB	Beta actin				Control		TGGTGATGGAGGAG-GTTTAGTAAGT	AACCAATAAAACC-TACTCCTCCCTTAA	6-FAM-ACCACCACCCAA-CACACAATAACAAACAC-ABHQ1
COL2A1	Collagen type 2	L10347			Control		TCTAACAATTATAA-ACTCCAACCACCAA	GGGAAGATGGG-ATAGAAGGGAATAT	6FAM-CCTTCATTCTAAC-CCAAT-ACCTATCCCACC-TCTAAA-BHQ1
1. *Cell cycle-related genes*									
SOCS1	Human suppressor of cytokine signalling-1	U88326	+304/+397	+350.5	Exon	70.85	GCGTCGAGTTCGT-GGGTATTT	CCGAAACCATCT-TCACGCTAA	6FAM-ACAATTCCGCTAA-CGACTATCGCGCA-BHQ-1
STAT1	Signal transducer and activator of transcription 1	AF182311	−444/−356	−400	Promoter	75.88	GCGTAGGATTCGG-AAGGGTTA	AACAAACCCCAA-ACCGAACA	6FAM-AACGACCCAACG-CGCTCGAAAA-BHQ1
CDKN2A	p16	NM_000077	+9/+69	+39	Prom/Exon	71.5	TGGAATTTTCGG-TTGATTGGTT	AACAACGTCCGC-ACCTCCT	6FAM-ACCCGACCCCGA-ACCGCG-BHQ1
ARF	p14/p19	AF082338	−203/−135	−169	Promoter	78	ACGGGCGTTTT-CGGTAGTT	CCGAACCTC-CAAAATCTCGA	6FAM-CGACTCTAAACCC-TACGCACGCGAAA-BHQ1
2. *Apoptosis-related genes*									
DR3	Death receptor 3	AB051850	+27/+95	+61	Prom/Exon	74.87	GCGGAATTACGAC-GGGTAGA	ACTCCATAACC-CTCCGACGA	6FAM-CGCCCAAAAACTT-CCCGACTCCGTA-BHQ-1
EDNRB	EDNRB, endothelin receptor B, CpG Island 2	NM_003991	+12/+104	+54	Prom/Exon	55.9	CGTTGGCGAGTT-ATGAGCGTT	TACCGCCCGC-AACCTCT	CGCGTAAATTTGAGTTA-TTTTTGAGCGTGGA
RASSF1A	Ras association (RalGDS/AF-6) domain family 1	NM_007182	+57/+121	+89	Prom/Exon	74.37	ATTGAGTTGCGGG-AGTTGGT	ACACGCTCCAACC-GAATACG	6FAM-CCCTTCCCAACGC-GCCCA-BHQ-1
BCL2	bcl-2 (B-cell lymphoma type 2)	NM_000633	+210/+293	+273	Exon	59.5	TCGTATTTCGGG-ATTCGGTC	AACTAAACGCA-AACCCCGC	6FAM-ACGACGCCGAAA-ACAACCGAAATCTACA-BHQ-1
TERT	Telomerase	AF325900	−223/−108	−165.5	Promoter	73.87	GGATTCGCGGGTA-TAGACGTT	CGAAATCCG-CGCGAAA	6FAM-CCCAATCCCTCC-GCCACGTAAAA-BHQ-1
DAPK1	Death-associated protein kinase 1	X76104	+137/+204	+170.5	Promoter	73.87	TCGTCGTCGTTT-CGGTTAGTT	TCCCTCCGAAAC-GCTATCG	6FAM-CGACCATAAACG-CCAACGCCG-BHQ1
DR-6	Tumor necrosis factor receptor superfamily, member 2	NM_014452	−426/−327	−476	Promoter	62.3	TCGGCGTTATTAC-GTGTGTTTT	TTCCAACCACTAC-CAACCACC	6FAM-CCTCCGCTACCC-AAACTAATCGACGAA-BHQ-1
TMS1	Apoptosis-associated speck-like protein (ASC)	NM_013258	+25/+105	+65	Prom/Exon	66.7	CGTTGGAGAATTT-GATCGTCG	CCCGTAACCC TCGCGCAA	6FAM-AGTTTAAGAAGTTT-AAGTTGAAGTTGTTGTCG-GTGTCG-BHQ1
PIG-7	LPS-induced TNF-alpha factor	NM_004862	−238/−143	−191	Promoter	58.8	GGTTGGGTTTAG-TTTTTGCGTT	GCGACGAAC-CGCGAAA	6FAM-CCACGCGCGATC-GAACCACTCT-BHQ-1
Bax	BCL2-associated X protein, isoform beta	NM_138762	+91/+169	+130	Prom/Exon	63.5	CGGGAGGTAGA-CGGGCG	CAACCCCAAAC-CGATAAAAAAA	6FAM-AGGGCGAGTTTT-TTCGTCGGTTCG-BHQ1
FADD	Fas (TNFRSF6)-associated *via* death domain	NM_003824	−138/−78	−100	Promoter	59.9	CGTTCGCGGCGTCG	GCGCATACGT-CCCTAACGA	6FAM-CCGCAACCGTTTC-CGCCCTAACTA-BHQ-1

3. Cell adhesion and metastasis genes									
ICAM1	Intercellular adhesion molecule 1 (CD54)	NM_000201	−166/−87	−126.5	Promoter	67.34	GGTTAGCGA-GGGAGGATGATT	TCCCCTCCGAAA-CAAATACTACAA	6FAM-TTCCGAACTAAC-AAAATACCCGAACCGAAA-BHQ-1
TIMP3	Tissue Inhibitor of metallinoproteinase 3	U33110	+1051/+1143	+1097	Exon	78	GCGTCGGAGGTTAA-GGTTGTT	CTCTCCAAAATT-ACCGTACGCG	6FAM-AACTCGCTCGCC-CGCCGAA-BHQ1
E-Cadherin	CDH1	L34545	−167/−72	−120	Promoter	68.5	AGGGTTATCGCGT-TTATGCG	TTCACCTACC-GACCACAACCA	6FAM-ACTAACGACC-CGCCCACCCGA-BHQ1
4. Angiogenesis and mismatch repair genes									
COX-2	PTGS-2	AF044206	−362/−217	−289.5	Promoter	62	CGGAAGCGTTCG-GGTAAAG	AATTCCACCG-CCCCAAAC	6FAM-TTTCCGCCA-AATATCTTTTCTTCTTC-GCA-BHQ1
MLH1	Mut L Homolog 1	NM_000249	−662/−575	−618.5	Promoter	68	CGTTATATATCGTTC-GTAGTATTCGTGTTT	CTATCGCCGCC-TCATCGT	6FAM-CGCGACGTCAA-ACGCCACTACG-BHQ1

non-recurrent tumours displayed a higher percentage of methylated tumours. In contrast, in *SOCS-1*, *BCL-2*, and *DAPK* the recurrent tumours displayed more frequent methylation. The methylation status of these genes was next used as grouping variable for a Kaplan–Meier analysis. The Kaplan–Meier analysis displayed a significant difference in the recurrence-free survival for *TIMP-3* ($P = 0.036$). The second best significance was found for methylation of *E-Cadherin* ($P = 0.075$) (Fig. 1). Interestingly, patients, in which *TIMP-3* was methylated, had a better disease-free survival than patients without. Furthermore, the association between tumour stage (pTa versus pT1) and tumour grading were used as grouping variable in a Kaplan–Meier analysis. Neither staging nor grading was significantly associated with recurrence free survival ($P < 0.05$, log-rank test).

4. Discussion

There is a clear need for development of prognostic markers in non-muscle invasive bladder cancer to provide risk-adjusted treatment and surveillance management. There is increasing interest in the utilisation of methylation markers, since promoter methylation-induced gene silencing has been proposed to be a third way in carcinogenesis according to Knudsons "two hit theory" [5]. In this study, we have screened 105 patients with primary non-muscle invasive bladder cancer for methylation at 20 genes with the Methylight technique. The genes investigated were linked to cell cycle regulation, apoptosis, cell adhesion and metastasis, or mismatch repair. In addition we investigated the *COX-2* gene and is linked to angiogenesis [24,25] and apoptosis [26]. We found frequent methylation in more than 70% of the tumour samples at *DR-3*, *ICAM-1*, and *COX-2*. Whereas *DR-3* and *ICAM-1* displayed intermediate or high methylation levels, *COX-2* only displayed a faint methylation. *STAT-1*, *p16CDKN2A*, *EDNRB*, *RASSF-1A*, *BCL-2*, *H-TERT*, *DAPK*, *TIMP-3*, and *MLH-1* displayed methylation in 10–69% of the tumours. *P14ARF*, *DR-6*, *TMS-1*, *PIG7*(*LITAF*), *BAX, FADD*, *E-Cadherin* displayed methylation in none of the tumours or only in single cases (<10%). When comparing methylation levels in recurrent versus non-recurrent tumours *SOCS-1*, *STAT-1*, *BCL-2*, *TIMP-3*, *E-Cadherin*, and *COX-2* displayed differences >10% in the fraction of methylated tumours, indicating that methylation of these genes might be linked to tumour recurrence. The Kaplan–Meier analysis and the log-rank test identified *TIMP-3* methylation to be significantly associated with recurrence-free survival. Our data demonstrate the prognostic relevance of methylation of *TIMP-3* for the recurrence of non-muscle invasive bladder carcinoma. We found that patients with methylation at *TIMP-3* had a

Table 2a
Percentage of methylated tumour samples, identifying four groups of genes according to the percentage of methylated tumours: 1. methylation in <10% of the tumour samples *p14ARF*, *DR-6*, *TMS-1*, *PIG7(LITAF)*, *BAX*, *FADD*, *E-Cadherin*; 2. methylation in 10–39% of the samples: *STAT-1*, *p16CDKN2A*, *H-TERT*, *DAPK*, *TIMP-3*, *MLH-1*; 3. methylation in 40–69% of the samples: *SOCS-1*, *EDNRB*, *RASSF-1A*, *BCL-2*; and 4. methylation in ⩾70%: *DR-3*, *ICAM-1*, *COX-2*

		Cell cycle regulatory genes				Apoptosis-related genes											Adhesion and metastasis genes				
		SOCS-1	STAT-1	p16CDKN-2A	P14ARF	DR-3	EDNRB	RASSF-1A	BCL-2	H-TERT	DAPK	DR-6	TMS-1	PIG-7 (LITAF)	BAX	FADD	ICAM-1	TIMP-3	E-Cadherin	MLH-1	COX-2
Non-malignant adjactant tissue	$n = 38$	9.9	2.6	0.0	0.0	89.5	50.0	42.1	10.5	2.6	5.2	0.0	2.6	0.0	0.0	0.0	100.0	42.1	34.2	0.0	76.3
PTa	$n = 81$	29.6	9.8	21.0	0.0	97.5	65.4	51.8	51.8	22.2	24.6	8.6	0.0	0.0	0.0	0.0	87.6	16.0	8.6	29.6	71.6
PT1	$n = 24$	45.8	4.1	4.1	8.3	100.0	83.3	95.8	50.0	12.5	20.8	0.0	0.0	0.0	0.0	0.0	100.0	33.3	8.3	58.3	91.7
Grade 1	$n = 23$	95.7	17.4	4.3	0.0	95.7	56.5	52.2	21.7	4.3	4.3	4.3	0.0	0.0	0.0	0.0	70.0	21.7	4.3	30.4	56.2
Grade 2	$n = 81$	35.8	11.1	12.3	0.0	98.7	24.6	61.7	59.3	24.7	23.4	4.9	0.0	0.0	0.0	0.0	91.3	17.2	9.9	35.8	80.2
Grade 3	$n = 1$	100.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.0	0.0	0.0	0.0	100.0	100.0	100.0	100.0	100.0
Total	$n = 105$	33.3	11.4	10.4	2.9	98.1	68.6	59.0	53.3	20.9	22.8	6.7	0.0	0.0	0.0	0.0	89.5	20.0	9.5	24.7	75.2

Table 2b
Quantitative analysis of methylation levels identifying five groups of genes according to the median PMR level: 1. PMR < 0.1%: *TMS-1*, *PIG7(LITAF)*, *BAX*, *FADD*; PMR 0.1–3.9%: *MLH-1*, *STAT-1*, *p16CDKN2A*, *p14ARF*, *DR-6*, *TIMP-3*, *COX-2*; 3. PMR 4%–19%: *H-TERT*, *DAPK*, *E-Cadherin*; 4. PMR 20–49%: *EDNRB*, *RASSF-1A*, *ICAM-1*; and 5. PMR 50–74.9%: *SOCS-1*, *BCL-2*, PMR ⩾ 75%: *DR-3*

		Cell cycle regulatory genes				Apoptosis-related genes											Adhesion and metastasis genes				
		SOCS-1	STAT-1	p16CDKN-2A	P14ARF	DR-3	EDNRB	RASSF-1A	BCL-2	H-TERT	DAPK	DR-6	TMS-1	PIG-7 (LITAF)	BAX	FADD	ICAM-1	TIMP-3	E-cadherin	MLH-1	COX-2
Non-malignant adjactant tissue	$n = 38$	0.3	0.1	0	0	57.5	1.8	14.2	31.3	0.3	1.8	0	0.5	0	0	0	43.7	0.3	0.3	0	
pTa	$n = 81$	55.5	0.2	1.9	0	272.8	29.5	25.5	58.6	10	5	2.1	0	0	0	0	50.1	1.9	4.7	3.6	4.5
pT1	$n = 24$	61.4	0.1	0.8	0.1	263.1	20.1	40.7	67.8	24.3	1.8	0.0	0.0	0.0	0.0	0.0	30.8	2.3	4.7	2.2	6.2
Grade 1	$n = 23$	0.8	1.5	5.4	0.0	248.9	14.3	2.1	34.6	0.3	47.3	2.1	0.0	0.0	0.0	0.0	45.7	1.5	0.2	5.4	5.7
Grade 2	$n = 81$	59.1	0.1	1.9	0.1	265.6	29.1	55.9	79.3	13.9	4.3	2.3	0.0	0.0	0.0	0.0	44.9	2.2	4.7	1.8	4.5
Grade 3	$n = 1$	45.0	0.0	0.9	0.1	332.7	38.2	149.3	56.3	2.2	3.3	0.1	0.0	0.0	0.0	0.0	58.6	1.4	6.9	32.0	11.1
Total	$n = 105$	55.5	0.1	0.9	0.1	265.6	28.9	29.7	63.1	11.4	4.4	2.1	0.0	0.0	0.0	0.0	46.8	2.0	4.7	4.7	5.7

Table 3a
Percentage of methylated samples in patients with recurrent or non-recurrent tumours in genes *SOCS-1*, *STAT-1*, *BCL-2*, *DAPK*, *TIMP-3*, *E-Cadherin*

		Cell cycle regulatory genes				Apoptosis-related genes											Adhesion and metastasis genes				
		SOCS-1	STAT-1	p16CDKN-2A	P14ARF	DR-3	EDNRB	RASSF-1A	BCL-2	H-TERT	DAPK	DR-6	TMS-1	PIG-7 (LITAF)	BAX	FADD	ICAM-1	TIMP-3	E-cadherin	MLH-1	COX-2
No recurrence	$n = 69$	30.4	14.5	8.7	2.8	98.5	68.1	68.1	46.4	18.8	21.7	7.2	0.0	0.0	0.0	0.0	87.0	30.4	13.0	33.3	69.6
Recurrence	$n = 26$	42.3	0.0	3.8	0.0	96.1	66.7	66.7	61.5	23.1	34.6	3.8	0.0	0.0	0.0	0.0	92.3	7.7	0.0	42.3	80.7

Table 3b
Difference in methylation levels between patients with recurrent and non-recurrent tumours

		Cell cycle regulatory genes				Apoptosis-related genes											Adhesion and metastasis genes				
		SOCS-1	STAT-1	p16CDKN-2A	P14ARF	DR-3	EDNRB	RASSF-1A	BCL-2	H-TERT	DAPK	DR-6	TMS-1	PIG-7 (LITAF)	BAX	FADD	ICAM-1	TIMP-3	E-cadherin	MLH-1	COX-2
No recurrence	$n = 69$	42.4	0.6	0.9	0.1	286	20.4	15.1	36.4	12.9	4.9	2.3	0	0	0	0	43.8	2.3	4.9	4.3	5.1
Recurrence	$n = 26$	92.9	0	33.3	0	207	23.4	81.7	101	22.1	3.7	0.3	0	0	0	0	48.3	1.1	4	1.4	2.2

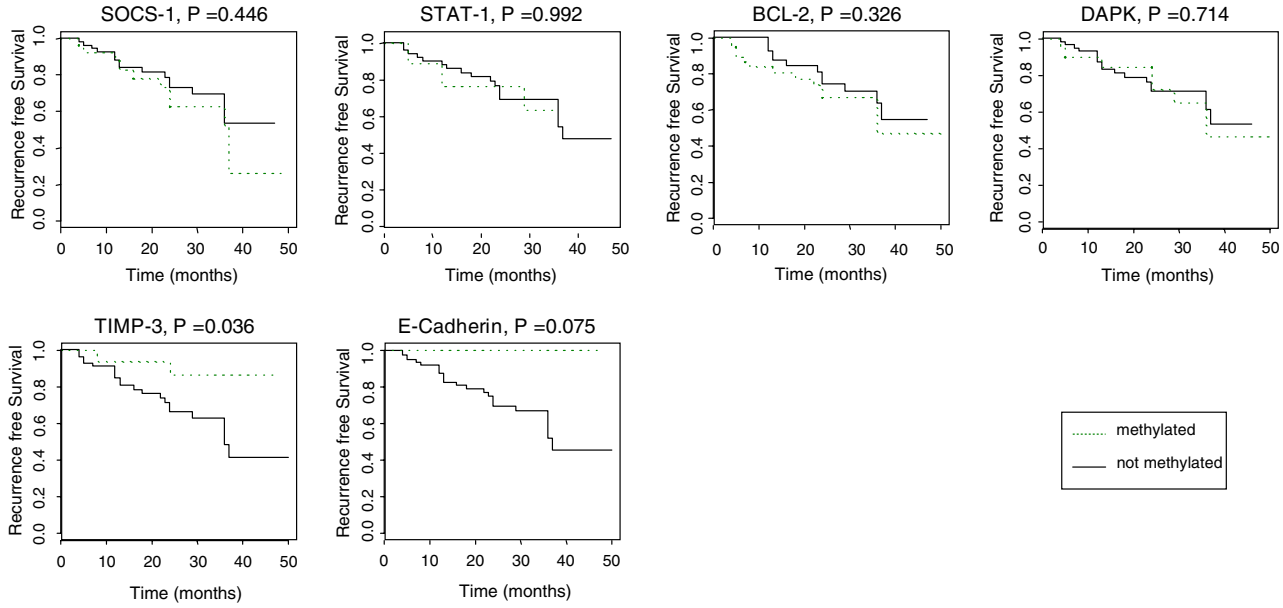

Fig. 1. Kaplan–Meier analysis for genes *SOCS-1*, *STAT-1*, *BCL-2*, *DAPK*, *TIMP-3*, *E-Cadherin*. The log-rank test displayed a significant difference in recurrence-free survival in *TIMP-3*.

significantly longer recurrence-free survival than patients without methylation at these loci. Our data indicate that these identified patients might be those who may be treated with less aggressive therapy because of lower risk for tumour recurrence. Even though we present a relatively large homogenic population that was treated according to a standardised protocol, it has to be stated that these data are exploratory. The number of recurrences in our study certainly is too small to draw definitive conclusions on the prognostic relevance for the individual patient.

There are an increasing number of reports on the prognostic value of methylation analysis in several cancers. Narayan and colleagues have described methylation status of *RARB* and *BRCA1* to predict worse prognosis in patients with cervix carcinoma [27]. Schneider-Stock *et al.* [28] have reported on *p16INK4a* alteration including methylation as independent prognostic marker for gastrointestinal stromal tumours. Roman-Gomez *et al.* [29] have described that *p*21 methylation status was an independent prognostic for recurrence-free survival in patients with acute lymphoblastic leukemia. In contrast to these authors who described an association of methylation of a certain gene with impaired clinical outcome, we could identify patients with a significantly better outcome than patients without methylation at these sites. Only few data are available on the prognostic value of gene methylation in human bladder cancer. Recently, Tada reported on the prognostic value of the numbers of methylated genes and on the prognostic value of methylation of *DAPK* for bladder cancer recurrence. They found a significant correlation with disease recurrence if 3 or more genes displayed methylation or if *DAPK* was methylated [9]. We confirmed an association between promoter methylation of *DAPK* and tumour recurrence. Since *DAPK* is more frequently methylated in patients who experience tumour recurrence, we could confirm the relevance of *DAPK* methylation in patients with pTa/pT1 bladder cancer. Although Kaplan–Meier analysis and the log-rank test did not reveal a significant difference in recurrence-free survival ($P = 0.714$). Regarding *E-Cadherin* Marayuma *et al.* [30] have described an association of methylation of *E-Cadherin* in muscle-invasive bladder carcinoma and survival. *E-cadherin* is a member of a family of transmembrane glycoproteins involved in intercellular adhesion. *E-cadherin* function is mediated by the interaction with the cytoplasmatic α-, β-, and γ-catenins. These catenins connect *E-cadherin* with the cytoskeleton. In model systems, loss of *E-cadherin* expression is associated with the gain of the invasive phenotype in tumours [31]. In bladder cancer, abnormal expression of *E-Cadherin* was associated with tumour invasion, lymph node status and clinical outcome [32–34]. There are increasing numbers of reports focusing on methylation of the *E-Cadherin* gene in bladder cancer describing methylation rates of 43–84%. In addition it was reported that promoter methylation is associated with gene silencing, which can be restored by treatment with 5-Aza-2′-deoxycitidine [8,10]. Marayuma described methylation rate of 29% [30], while there is a notable difference in the percentage of methylated samples between our data and the reports by other authors. One explanation might be the different techniques used. Bornman and Ribeiro-Filho used methylation-specific PCR (MSP). Both techniques, Methylight and MSP, are

based on the amplification of fully methylated DNA-strands. In contrast to MSP, Methylight is a quantitative technique that enables the calculation of the percentage of fully methylated DNA strands. Another explanation may be the focus on patients with primary pTa/pT1 tumours, who did not receive adjuvant intravesical treatment. Our data are an interesting complement to the findings of Marayuma indicating that the same alteration can result in a more favourable outcome in patients with non-invasive disease; and in contrast a less favourable outcome in patients with an invasive disease. In patients with an advanced tumour, the loss of cell–cell adhesion might result in a more aggressive tumour type with the ability to metastasise. In contrast, in a non-invasive tumour, a decrease in cell–cell interactions may result in a lack of ability for setting an implantation metastasis in another site of the bladder, that could be the source of a recurrent tumour [35] and might therefore result in a longer recurrence-free survival.

TIMP-3 is a member of the family of secreted inhibitors that blocks the activity of metallomatrix proteins (MMPs), therefore regulating cell growth; invasion and migration; tumour progression; and development of metastasis [36]. To our knowledge, no data are available on the methylation status of *TIMP-3* in bladder carcinoma. Regarding other tumour entities, Esteller and colleagues have reported a frequency of 5% (liver carcinoma) to 76% (kidney carcinoma) of methylation in several types of tumour samples [14]. Regarding pancreatic tumours, Wild have described frequent methylation-associated silencing of *TIMP-3* [36]. Zochbauer-Muller *et al.* [37] have reported on a frequency of 26% methylated tumours in non-small cell lung carcinoma without finding any relation to clinical outcome.

We present a comprehensive analysis on prognostic relevance of gene methylation markers in non-muscle invasive bladder cancer. In large series of patients with primary pTa/pT1 bladder cancer, we found that methylation of *TIMP-3* was associated with a more favourable outcome. Our data strongly support the usefulness of methylation markers as a prognostic marker in patients with non-muscle invasive bladder cancer.

Conflict of interest statement

None declared.

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