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ORIGINAL ARTICLE – CANCER RESEARCH

A three-gene methylation marker panel for the nodal metastatic risk assessment of muscle-invasive bladder cancer

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Abstract

Purpose In this study, we aimed to identify a DNA methylation pattern suitable for prognosis assessment of muscle-invasive bladder cancer and to investigate metastasis-associated processes regulated by DNA methylation.

Methods Genome-wide methylation analysis was performed on 23 muscle-invasive bladder tumors by microarray analysis. Validation was performed by the qAMP technique in two different patient cohorts ($n = 32$ and $n = 100$). mRNA expression was analyzed in 12 samples. Protein expression was determined using tissue microarrays of 291 patients. Bladder cancer cell lines T24 and 253JB-V were used for functional analyses.

Results Microarray analyses revealed KISS1R, SEPT9 and CSAD as putative biomarkers with hypermethylation in node-positive tumors. The combination of the three genes predicted the metastatic risk with sensitivity of 73% and specificity of 71% in cohort 1, and sensitivity of 82% and specificity of 54% in cohort 2. mRNA expression differences were detected for KISS1R ($p = 0.04$). Protein expression of KISS1R was significantly reduced ($p < 0.001$). Knockdown of SEPT9v3 resulted in increased cell migration by 28% ($p = 0.04$) and increased invasion by 22% ($p = 0.004$). KISS1R overexpression resulted in decreased cell migration (25%, $p = 0.1$).

Conclusions We identified a methylation marker panel suitable to differentiate between patients with positive and negative lymph nodes at time of cystectomy. This enables a risk assessment for patients who potentially benefit from extended lymph node resection as well as from neoadjuvant chemotherapy and could improve the survival rates. Furthermore, we examined the impact of putative markers on tumor behavior. Hence, KISS1R and SEPT9 could represent a starting point for the development of novel therapy approaches.

Keywords Muscle-invasive bladder cancer · Methylation · Biomarker · Metastasis

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Background

Bladder cancer (BC) is the ninth most common cancer world wide and one of the most expensive human malignancies to manage (Svatek et al. 2014). Around one-third of patients have muscle-invasive bladder cancer (MIBC), which has an ominous prognosis. The 5-year survival rate can be less than 50%. A significant part of the patients develops metastases before or after radical treatment (Stein et al. 2006). In the presence of regional or distant metastases, the 5-year survival rate drops to 33% and 6%, respectively (Williams and Stein 2004). Radical cystectomy is the standard curative treatment for patients with localized MIBC, including bilateral lymph node removal. Depending on tumor extension and lymph node involvement, a perioperative cisplatin-based chemotherapy is indicated (Witjes et al. 2014). Improvements in survival can be achieved by systemic cisplatin-based chemotherapy, in either the neoadjuvant or adjuvant setting (Niegisch et al. 2013; Booth and Tannock 2015), or even early radical treatment for aggressive non-muscle-invasive cancers. Perioperative chemotherapy, on the other hand, is far from nontoxic and the consecutive survival benefit can only be described as modest. Stratifying patients for or against perioperative systemic treatment currently depends on histopathological examination which may lead to both over- and under-treatment of MIBC, in particular when tumor samples from transurethral resection are used. There certainly is an urgent need to develop biomarkers, which can be used to supplement histological parameters to guide and to individualize decision-making process.

Tumor progression from localized to metastatic disease is thought to represent a stepwise acquisition of molecular traits arising from genetic or epigenetic alterations (McAllister and Weinberg 2014). Of molecular alterations, DNA methylation appears to be a particularly promising biomarker and therapeutic target, as alterations occur early during carcinogenesis, which can be reversed pharmacologically throughout the entire genome (Esteller 2007). Whilst various reports have profiled changes in DNA methylation at a global epigenome and candidate gene-specific level, little is known about changes during metastatic progression (Dhawan et al. 2006; Phe et al. 2009). Authors have demonstrated that increased DNA methylation is associated with aggressive tumors and higher recurrence rates, progression and cancer-specific mortality.

With this in mind, we sought to profile DNA methylation in lymph node-positive BC to detect reliable biomarkers for the metastatic risk. We undertook a genome-wide assessment and investigated the impact of putative methylation-based markers in the metastatic processes.

Methods

Patient samples

Genome-wide analysis to discover methylation differences was conducted using 23 BC specimens from the Department of Urology, Jena University Hospital. Methylation status of candidate genes was examined in 32 specimens. Data validation was performed on an external patient cohort of 100 formalin-fixed, paraffin-embedded BC samples provided by the German "Arbeitsgemeinschaft Urologische Onkologie" (AUO AB 5–95) cohort treated by adjuvant chemotherapy (Lehmann et al. 2006). mRNA expression was analyzed in 12 BC tissue samples from Jena cohort. Detailed cohort description can be found in Table 1. Protein expression was determined using TMAs of 291 patients provided by the Departments of Urology and Pathology, University Hospital Dresden, and the Institute of Pathology, University Hospital Erlangen. Patients from Jena and AUO cohorts did not receive neoadjuvant therapy before cystectomy. In the TMA series, 18 patients have been pretreated by neoadjuvant chemotherapy.

The study was approved by the local ethics committee.

CpG island microarray and data analysis

Eight micrograms sonicated DNA was enriched for methylated fragments using the methylated DNA immunoprecipitation (MeDIP) as described elsewhere (Weber et al. 2005). The methylated fraction and an untreated reference fraction were differentially fluorescent labeled (Genomic DNA Enzymatic Labeling Kit, Agilent) and hybridized on the 244K Human CpG Island (CpGI) Microarray (Agilent) according to the instructions. Microarrays were scanned (High-Resolution C Scanner, Agilent). Image analysis and data extraction were performed using Agilent Feature Extraction software (v9.5.3). Methylation levels (log2ratios) were calculated by Genomic Workbench software 5.0 (Agilent). We defined probes within the highest and lowest quintiles (20%) as hyper- or hypomethylated. Genes with hypermethylated promoter region in lymph node-positive patients were selected as candidate genes for further validation.

DNA methylation validation

Microarray data validation was conducted using the qAMP method described by Oakes et al. (2006) (Supplement). qPCR analysis was performed using GoTaq® qPCR Master Mix (Promega) and StepOnePlus™ device (Life technologies). SNRPN, an imprinted genomic region, was used as reference.

Table 1 Patient characteristics of discovery set (microarray analysis), test set (qPCR) and validation set (qPCR)

	Discovery set (microarray analysis) $n=23$	Patient cohort 1 (test set) $n=32$	Patient cohort 2 (validation set) $n=100$
Gender, n (%)			
Male	21 (91.3)	29 (90.6)	82 (82.0)
Female	2 (8.70)	3 (9.38)	18 (18.0)
Age, mean ± STD	67.0 ± 9.17	68.5 ± 7.93	60.2 ± 7.97
pT, n (%)			
2	6 (26.1)	6 (18.8)	2 (2.0)
3	12 (52.2)	19 (59.4)	84 (84.0)
4	5 (21.7)	7 (21.9)	12 (12.0)
Lymph node, n (%)			
Negative	14 (60.8)	21 (65.6)	50 (50.0)
Positive	9 (39.1)	11 (34.4)	50 (50.0)
Progression			
Yes	11 (47.8)	14 (43.8)	50 (50.0)
No	11 (47.8)	16 (50)	50 (50.0)
Unknown	1 (4.35)	2 (6.25)	0
Survival			
Yes	10 (43.5)	17 (53.1)	55 (55.0)
No	12 (52.2)	13 (40.6)	45 (45.0)
Unknown	1 (4.35)	2 (6.25)	0
Follow-up (month) mean	17.7 (2–60)	27.5 (2–60)	31.07 (2–95)

mRNA expression analysis

Total RNA was extracted using ReliaPrep™ RNA Cell Miniprep System (Promega). RNA integrity was determined by 2100 Bioanalyzer (Agilent). Total RNA was reverse transcribed by GoScript™ Reverse Transcription System (Promega). ATP5B was used as reference gene.

Tissue microarrays and immunohistochemistry for KISS1R

TMA slides were immunohistochemically stained using the VECTASTAIN® ABC universal Kit (Vector Laboratories) according to the manufacturer's instructions. KISS1R primary antibody (AXOR12 375–398, Phoenix Pharmaceuticals) was diluted 1:500. Placenta tissue served as positive control. Incubation without primary antibody served as negative control. KISS1R staining was evaluated by light microscopy. Staining intensity was assigned into 0 = no staining, 1 = weak, 2 = intermediate, 3 = strong. Staining score was calculated as staining intensity × percentage of positive cells.

KISS1R overexpression and SEPT9v3 knockdown by transient transfection

For KISS1R overexpression, 8×10^4 T24 cells were transfected with 100 pg of TrueClone® cDNA-Vector and MegaTran 1.0 transfection medium (OriGene Technologies, Inc.). For negative control, cells were incubated only in the presence of transfection medium. Cells were transfected in duplicates. For SEPT9v3 knock down, 1×10^5 253J-BV cells were incubated with 75 pmol SEPT9 siRNA (MISSION Predesigned siRNA, Sigma). All Stars siRNA (Qiagen) served as negative control. DharmaFect 1 (Fisher Scientific) was used as transfection medium. All transfections were conducted for 48 h in three independent experiments. Transfection efficiency was examined by qRT-PCR.

Cell proliferation and migration assay

Changes in proliferation and migration were monitored by the Real-Time Cell Analyzer System (ACEA). For proliferation measurements, 1×10^4 cells were suspended in complete medium and added to E-Plate wells. Impedance changes, which correlate with the confluence level, were measured at the bottom side of the well. Results were given as cell indices which were measured every 30 s for 4 h and afterwards every 30 min for 48 h. Analysis was run in duplicates at 37 °C/5%CO_2. Cell-free complete medium was used as blank value. For migration analysis, transfected cells were seeded into CIM Plates. 2×10^4 transfected cells were suspended in serum-free medium and seeded into the upper chamber. The lower chamber contained complete medium with FBS as migration attractant. Impedance changes were measured at the bottom side of the membrane. Cell migration was monitored for 20 h every 30 s.

Invasion assay

Invasion assay was performed using BioCoat™ BD Matrigel™ Invasion Chamber (Becton Dickinson). The lower compartment contained 750 µl medium with 10% FBS as a chemoattractant. In the upper chamber, 5×10^4 cells in 500 µl serum-free medium were seeded and incubated at 37 °C/5% CO_2 for 24 h. Invaded cells at the bottom side of the filter membrane were stained with 0.1% crystal violet. The dye was extracted with 10% acetic acid. The extracted dye amount is proportional to the cells' invasiveness and was colorimetrically measured at 595 nm.

Statistical analysis

Statistical analyses were performed using SPSS 19.0. Non-parametric continuous data were analyzed using Mann–Whitney *U* test.

Sensitivity, specificity, and positive and negative predictive values were calculated, and ROC curve analysis and contingency tables were used for cut-off determination. Progression-free survival (PFS) and overall survival (OS) were analyzed by Kaplan–Meier analysis and log-rank test. *p* values < 0.05 indicated statistical significance. All tests were two sided.

Results

Genome-wide methylation differences of MIBC in correlation to the lymph node status

The patient and tumor characteristics are shown in Table 1. Using microarray analysis, we identified 67 CpGIs distinguishing lymph node-positive (pN+) from lymph node-negative (pN−) MIBC (FDR < 0.01, $p < 0.01$). Of these, 24 CPGIs were hypermethylated and 43 CPGIs were hypomethylated in lymph node-positive MIBCs when compared to the lymph node-negative cohort (Fig. 1, Supplement).

Functional annotation analysis performed using DAVID Bioinformatics Resources 6.7 revealed that 30% of the CpGI-associated genes are related to DNA binding and transcriptional function. Further, 15% are encoding for developmental proteins and 6% play a role during developmental process (Dennis et al. 2003).

DNA methylation validation and establishment of a methylation panel for the metastatic risk assessment

For further evaluation, we selected KiSS1 receptor (KISS1R), Septin9 (SEPT9) and cysteine sulfinic acid decarboxylase (CSAD) given their role in urothelial carcinogenesis and their ability to identify BC patients with nodal metastases. For each gene, significantly increased promoter

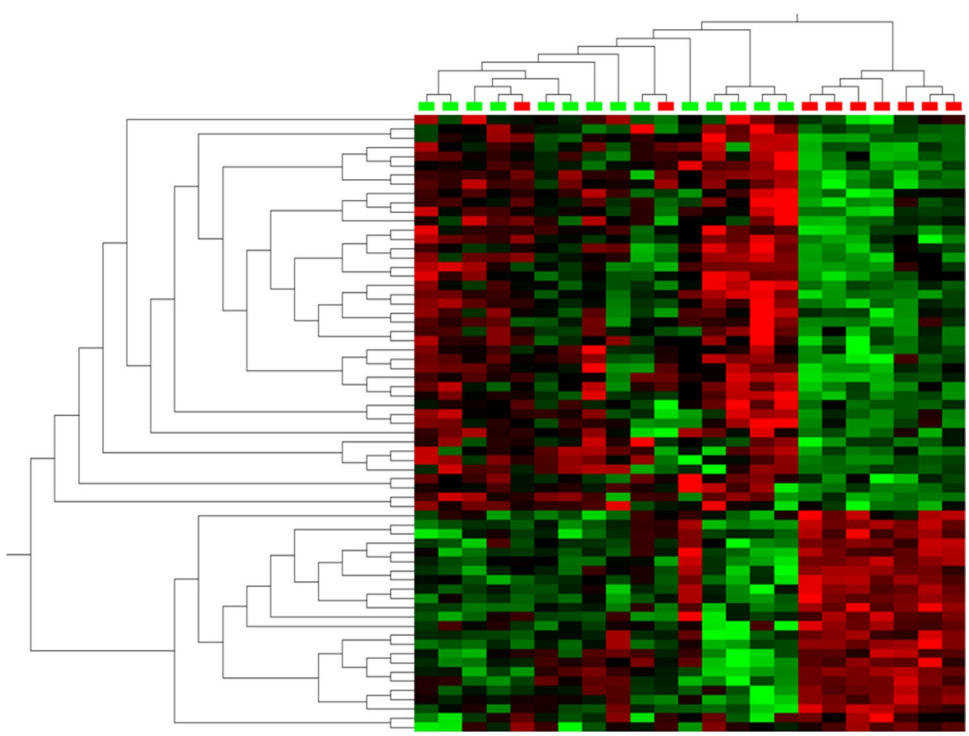

Fig. 1 Microarray heat map. Statistically significant (*p* value < 0.05; FDR < 0.05) differences in CpG Island methylation level of metastatic ($n = 9$, red) and non-metastatic ($n = 14$, green) primary bladder tumors

methylation was seen in lymph node-positive BC patients (Table 2). Neither gene could robustly stratify metastases by univariate analysis. The combination of loci improved risk assessment: patients having two or three loci with methylation above the corresponding cut-off value were assigned to a positive test result indicating a higher risk at the development of metastasis; patients with zero and one hypermethylated gene were assigned to a negative test result and hence to a lower metastatic risk.

ROC curve analysis revealed that the three-gene panel application improved the assessment of positive lymph nodes with a sensitivity of 73% and specificity of 71%, AUC = 0.72 (Fig. 2a). We also tested the combination of CSAD and KISSR alone excluding SEPT9 which showed the lowest AUC. This resulted in higher sensitivity (82%) and lower specificity (42%) and, therefore, in a lower AUC (0.62). The three-gene panel reliability was further validated on an enlarged, independent patient cohort ($n = 100$) and resulted in an increased sensitivity of 82% and slightly decreased specificity of 54%, (AUC = 0.68; Fig. 2b).

The panel revealed an association between the assigned test result and the PFS rate of the MIBC patients. The Kaplan–Meier curves indicated higher PFS rates for patients with negative test results in both patient cohorts (Fig. 3). However, log-rank test indicated no significance for PFS ($p = 0.18$ and 0.25 in cohort 1 and cohort 2, respectively).

mRNA and protein expression in BC tissue

Significant expression differences ($p = 0.04$) were detectable for KISS1R (Fig. 4a). KISS1R expression levels showed inverse proportionality to DNA methylation. According to the mRNA expression results for KISS1R, this candidate gene was selected for immunohistochemical staining on TMAs in two independent cohorts ($n = 291$). In accordance

Table 2 Relative DNA methylation of candidate genes validated in the test set with corresponding cut-off values to determine lymph node status

Candidate gene	Relative DNA methylation level		Cut-off	Sensitivity	Specificity	AUC
	pN0 ($n = 21$)	pN+ ($n = 11$)				
CSAD	1.68	1.73	1.69	73%	67%	0.70
SEPT9	1.73	1.75	1.73	64%	52%	0.58
KISS1R	1.71	1.84	1.72	73%	57%	0.67

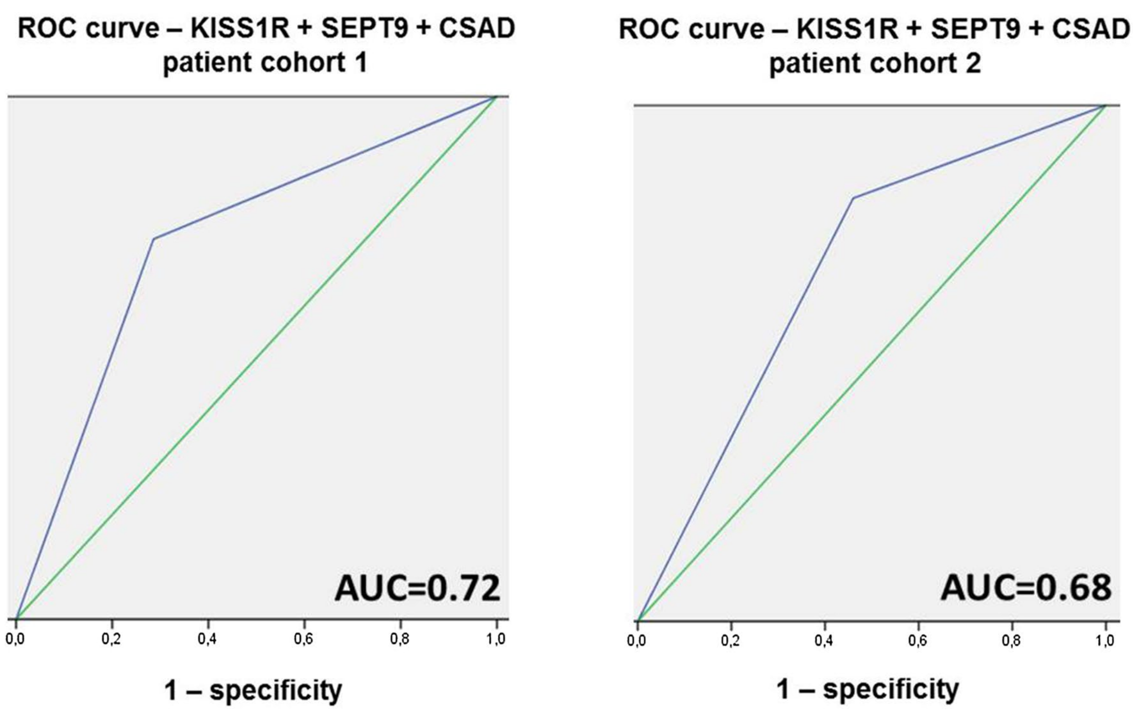

Fig. 2 ROC curve analysis of the gene panel combining KISS1R, SEPT9 and CSAD methylation for the assessment of the metastatic risk of primary tumors in two independent sample cohorts. The combination of the three candidate genes resulted in a (**a**) sensitivity of 73% and specificity of 71% in patient cohort 1 (AUC = 0.72) and **b** sensitivity of 82% and specificity of 54% in patient cohort 2 (AUC = 0.68)

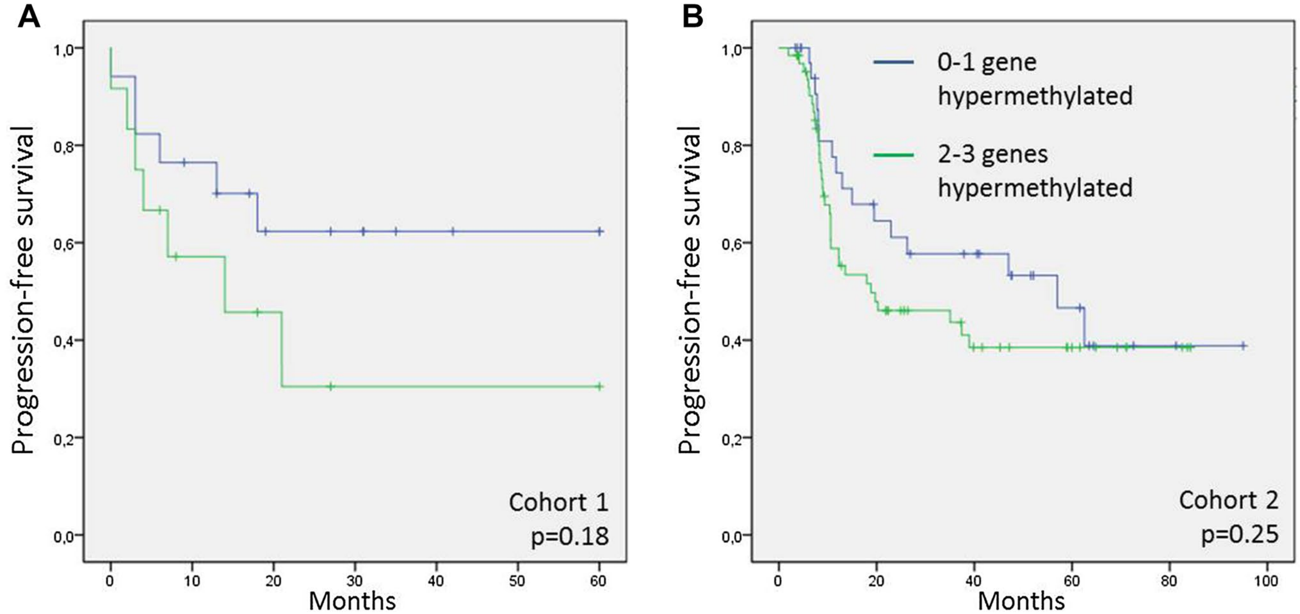

Fig. 3 The three-gene panel comprising KISS1R, SEPT9 and CSAD promoter methylation is associated with the progression rate of MIBC in two independent patient cohorts. The blue line represents patients with lower candidate gene methylation (0–1 gene hypermethylated) which was assigned to a negative test result showing better progression-free survival in both patient cohorts. The green line represents patients with 2–3 hypermethylated genes and a positive test result. (+) indicates censored data

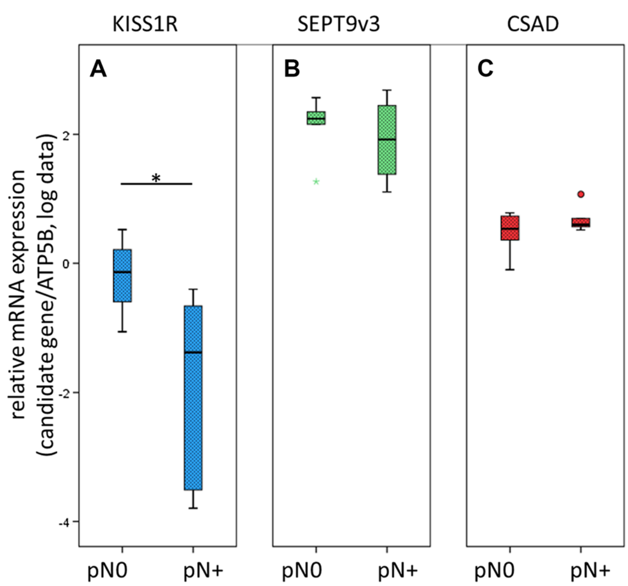

Fig. 4 Relative mRNA expression level of candidate genes in BC tissue. Box plots indicate the distribution of the relative mRNA expression levels of **a** KISS1R, **b** SEPT9 and **c** CSAD in lymph node-negative (pN0) and lymph node-positive (pN+) MIBC

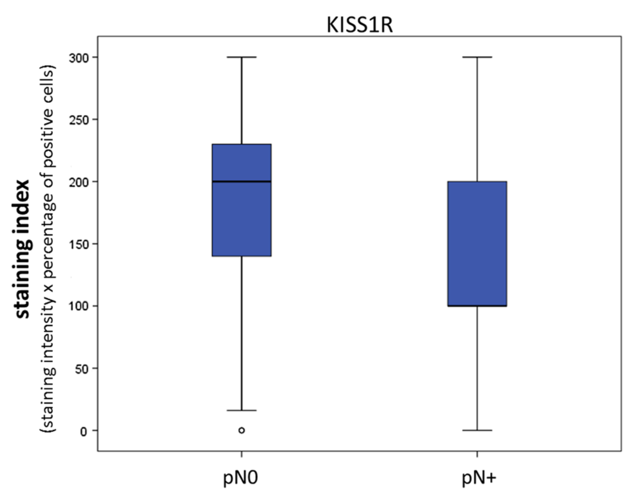

Fig. 5 Relative protein expression of KISS1R in BC tissue. Box plots present the distribution of the staininig indexes of KISS1R protein assessed by tissue microarrays in lymph node-negative (pN0) and lymph node-positive (pN+) MIBC. Staining intensity was assigned into 0 = no staining, 1 = weak, 2 = intermediate, 3 = strong. Staining score was calculated as staining intensity × percentage of positive cells

with the mRNA results, a significantly lower protein level ($p<0.001$) was detected in pN+ patients (Fig. 5).

For SEPT9, seven transcript variants are described. (Connolly et al. 2011) Literature research and transcript variant analysis revealed that SEPT9 transcript variant 3 (SEPT9v3) is negatively regulated by promoter methylation (data not shown). However, SEPT9v3 was only slightly reduced. Statistical analysis revealed no significant expression differences for SEPT9v3 and CSAD (Fig. 4b, c).

Impact of altered mRNA expression on metastasis-specific processes in BC cell lines

KISS1R overexpression in T24 cells was confirmed by qRT-PCR. No KISS1R amplification was detectable in untransfected cells ($C_T > 50$) while cDNA transfection for 48 h resulted in amplification (0.25 ng cDNA: $C_T = 17.6$; 0.75 ng cDNA: $C_T = 11.9$). Functional analysis revealed that KISS1R overexpression has no impact on T24 cell proliferation ($p = 0.5$). Cell migration analyses resulted in a decreased migration rate by 25%. However, differences were not significant ($p = 0.1$). The cells' invasion potential was decreased by 5% ($p = 0.7$) (Fig. 6a).

To assess the influence of changed SEPT9v3 expression, 253J-BV cells were selected as they showed highest SEPT9v3 expression. Following 48-h siRNA transfection, SEPT9v3 mRNA expression was significantly reduced by 25% in 253J-BVcells compared to cells transfected with negative control siRNA ($p = 0.02$) (data not shown). Functional analysis resulted in 10% increased cell proliferation ($p = 0.5$), 28% higher cell migration ($p = 0.04$) and 22% higher cell invasion potential ($p = 0.004$) (Fig. 6b).

Discussion

It is well known that altered epigenetic control of gene expression is important in tumor development and progression (Jones and Baylin 2002). Changes in DNA methylation are detectable in precancerous stages and correlate with the aggressiveness of tumors (van Hoesel et al. 2013). In this study, we focused on epigenetic events within lymph node-positive MIBC, with regard to defining a prognostic panel to stratify the use of neoadjuvant chemotherapy. Genome-wide analysis revealed methylation differences associated with the presence of lymph node metastases at the time of cystectomy. This pathological feature is one of the strongest predictors of cancer-specific death and is, therefore, used to guide chemotherapy (Margulis et al. 2008).

Our microarray results served as selection tool of putative prognostic biomarkers. We focused on aberrantly methylated genes in node-positive primary tumors. Due to the complex metastatic processes, it seems unlikely that single biomarkers allow a reliable risk assessment. Hence, we defined a marker panel to combine the three candidate genes. In the validation set of 100 independent patients, this combined panel correctly identified the regional lymph node status in

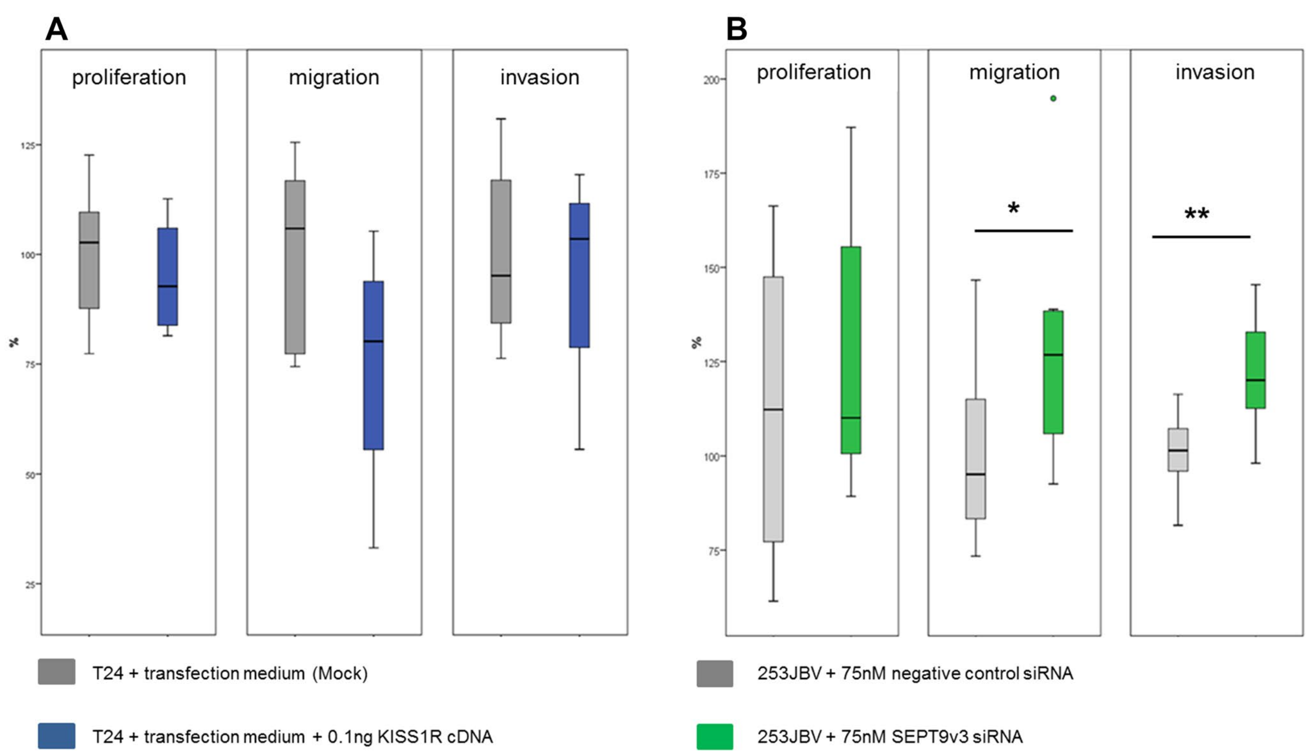

Fig. 6 Impact of the candidate genes KISS1R and SEPT9v3 on cell proliferation, migration and invasion in BC cells. **a** Increased KISS1R expression by cDNA overexpression had no significant influence on cell proliferation, migration and invasion. Proliferation and invasion were reduced by 5%. Cells showed a reduced migration potential by 25%. **b** Decreased Sept9v3 expression had no influence on cell proliferation ($p = 0.5$) but increased cell migration ($p = 0.04$) and cell invasion ($p = 0.004$)

around 70% of patients. Our data suggest that the combined DNA methylation level of KISS1R, SEPT9 and CSAD may be a suitable panel for the metastatic risk assessment of MIBC. To use this marker panel to select patients for neoadjuvant therapy, the presented results have to be validated in tumor samples obtained by transurethral resection.

A genetic study by Smith *et al*. described a RNA expression panel of 20 genes predicting lymph node metastases of high-risk BC patients who would benefit from neoadjuvant chemotherapy (Smith et al. 2011). Recently, this study has been disproved by van Kessel et al. who validated the signature in MIBC samples and did not find any correlation between these expression levels and node-positive disease (van Kessel et al. 2017). The observations make us clear that biomarker panels need repeated independent validation approaches and also reflect the heterogeneity and complexity of metastatic processes requiring well-defined clinical parameters for patient cohort selection.

In our methylation panel, we used Kaplan–Meier analyses to reveal further its prognostic power. The data showed that patients with few (0–1 of 3) hypermethylated genes had less progression events and longer PFS compared to those with more (2–3 of 3) hypermethylated genes. Even the difference failed to reach statistical significance, the plots suggest that this also reflects sample heterogeneity rather than a lack of biological differences.

Our candidates have an interesting biological potential. KISS1R is a G protein-coupled receptor involved in the inhibition of tumor growth and spread. Activation by its endogenous ligand results in intracellular calcium release and induction of cell differentiation or apoptosis (Muir et al. 2001). It mediates MAPK signaling activation and may contribute to decreased MMP expression via NFκB degradation leading to metastasis (Ji et al. 2013). On the functional level, we were able to show a trend to reduced cell migration when KISS1R is expressed in T24 cells. In terms of our methylation results, these data indicate that KISS1R hypermethylation and consequentially its reduced expression could contribute to tumor cell spread within the metastatic process. Similar results were found in thyroid and breast cancers where increased KISS1R expression leads to inhibition of tumor cell migration (Stathatos et al. 2005; Olbrich et al. 2010). In addition to noticeable differences in DNA methylation, expression analysis on mRNA and protein level revealed significant differences between lymph node-positive and -negative tumors for KISS1R. Especially, the significance on both expression levels underlines the suitability of KISS1R for prognosis assessment.

The physiological function of SEPT9 is the regulation of the cytoskeleton structure during cell division. SEPT9 is involved in several cellular processes such as vesicle transport, chromosome segregation, actin organization and cell cycle arrest, and is described to have tumor-suppressive and oncogenic functions (Sorensen et al. 2002; Kremer et al. 2007). SEPT9 hypermethylation is a promising biomarker in colorectal cancer (Toth et al. 2011). We performed a specific inhibition of SEPT9v3 in BC cells which resulted in a significant increase of cell migration and invasion. These findings underline that the expression may inhibit migration and invasion (which is attenuated when silenced by promoter hypermethylation). So far, there are no data available concerning SEPT9v3 in tumor cell behavior. However, other results indicate that disturbances in the interaction of several SEPT9 isoforms contribute to changes in cell function and behavior. For SEPT9v3, only a trend to lower expression in metastatic tumors could be detected. Due to low RNA integrity of primary tumor tissues, only 30% of the total RNA could be analyzed. Analyses of more BC samples could provide better information about the expression levels in MIBC. Protein expression analyses have not been performed due to a lack of specific antibody for the desired transcript variant.

CSAD is mainly expressed in the liver where its function is the decarboxylation of cysteine sulphinic to hypotaurin in taurin synthesis which has anti-inflammatory and immunoregulatory effects (Hope 1955; Schuller-Levis and Park 2004). No correlation between CSAD and tumor progression has been described. As no expression differences were detectable for CSAD in patient material and BC cell lines, the protein level of CSAD was not assessed. Functional analysis of CSAD failed due to low transfection efficiency.

We have recognized that our study is limited by the heterogeneity of our sample cohorts and retrospective character. To enlarge our validation set, we used samples originating from a multicenter trial aiming at the assessment of effects of adjuvant chemotherapy on survival rates of MIBC patients with ≥ pT3 and/or pN + stage reflecting an extremely high-risk nature that may explain the worse specificity of cohort 2 (Lehmann et al. 2006). Of note, all these patients received adjuvant chemotherapy with two different regimens within this trial. Therefore, analyses of PFS and OS are biased by the selection of high-risk patients, the absence of a standardized lymph node dissection and the potential influence of therapy.

Conclusion

We have identified a putative prognostic methylation marker panel that allows the differentiation between MIBC patients with positive and negative lymph node status at the time of cystectomy. This might enable a risk assessment for patients who benefit from extended lymph node dissection as well as neoadjuvant chemotherapy and could finally improve the survival rates of MIBC. The additional prognostic value of the methylation panel has to be tested prospectively including clinical and histopathological parameters. Furthermore, we examined the impact of putative prognostic markers on tumor cell behavior. Hence, KISS1R, SEPT9 and CSAD could represent a starting point for the development of novel therapy approaches in MIBC.

Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Human and animal rights statement This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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