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ORIGINAL ARTICLE – CANCER RESEARCH

MicroRNAs in tumor samples and urinary extracellular vesicles as a putative diagnostic tool for muscle-invasive bladder cancer

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Abstract

Purpose The identification of biomarkers characterizing the invasive potential of bladder cancer could enhance the clinical management of individual patients and therefore improve prognosis. The aim of this study was to define a miRNA panel in tumor tissues as well as in urinary extracellular vesicles (EVs) for discriminating muscle-invasive bladder cancer (MIBC) from non-muscle-invasive bladder cancer (NMIBC).

Methods miRNA expression was analyzed in 24 formalin-fixed, paraffin-embedded (FFPE) tumor samples by microarray analysis and was further validated by qRT-PCR in 56 FFPE tumor samples as well as in 37 urinary EV samples.

Results Microarray analysis revealed 63 miRNAs that were significantly differentially expressed ($P < 0.05$) between tissues from MIBC and NMIBC tumors. Five selected miRNAs (miR-146b-5p, miR-155-5p, miR-138-5p, miR-144-5p, and miR-200a-3p) were validated by qRT-PCR. The expression of all except miR-144-5p was significantly associated with high tumor grade. In urinary EVs, a different expression was verified for miR-146b-5p ($P = 0.004$) and miR-155-5p ($P = 0.036$), which exhibited significantly higher expression in urinary EVs from patients with MIBC.

Conclusions miRNAs are promising biomarkers for the identification of invasive bladder carcinomas. Tissue samples as well as urinary EVs may serve as sources for miRNA analysis. This method, in addition to histopathology, could provide a new diagnostic tool and facilitate individual therapeutic decisions to select patients for early cystectomy.

Keywords Bladder cancer · Muscle-invasive bladder cancer · microRNA · Extracellular vesicle · Urine

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Introduction

Bladder cancer is the ninth most common cancer worldwide. The outcome is mainly defined by tumor invasiveness: patients with non-muscle-invasive bladder cancer (NMIBC; accounting for ~ 70% of bladder tumors) have a low risk of tumor-related death (90% 5-year survival rate) despite a high recurrence rate (Babjuk et al. 2017; Knowles and Hurst 2015). In contrast, the 5-year survival rate of patients suffering from muscle-invasive bladder cancer (MIBC) is strongly correlated with pT stage and can be 40% or even lower for patients with locally advanced disease because of a high risk of distant dissemination. To date, no reliable biomarkers are available that allow noninvasive differentiation of MIBC from NMIBC. Understanding the molecular mechanisms of invasive tumor phenotypes will improve the identification of new specific diagnostic biomarkers on the one hand and the development of new therapeutic strategies on the other hand. Complex molecular analyses elucidated that MIBC is characterized by a distinct molecular pathway including specific genomic alterations different from those in NMIBC (Knowles and Hurst 2015). MicroRNAs (miRNAs) are an important group of key players in tumor cell migration and dissemination. The exact function of miRNAs in the progression and dissemination of MIBC, however, is not completely understood.

miRNAs are small, noncoding RNAs that influence biological processes, including proliferation, differentiation, apoptosis and migration (Bartel 2004). The sequence-specific pairing of miRNAs with their target mRNAs leads to mRNA degradation or translational repression, whereby miRNAs modulate the expression of their target genes. Deregulation of miRNA expression has been described in many tumors and is associated with alterations in invasion, migration and epithelial–mesenchymal transition (EMT), which can facilitate the invasiveness of MIBC (Le et al. 2010). We previously reported that bladder cancer cell lines are characterized by a specific invasion-associated miRNA expression signature, supporting a crucial role of miRNAs in promoting an invasive phenotype (Baumgart et al. 2017a). In addition, we analyzed the miRNA profile of extracellular vesicles (EV) secreted by these cells.

EVs are small membraned vesicles with a size of 30–150 nm that are secreted by all cell types. EVs transfer molecules from parental to recipient cells and therefore are involved in cell–cell communication (Escola et al. 1998; Mellman 1996). EVs regulate physiological and pathophysiological processes such as tumor development, the generation of an invasion-supporting environment and the formation of a premetastatic niche (Chen et al. 2014; Cho et al. 2012; Corrado et al. 2013; Fevrier and Raposo 2004). In this regard, miRNAs incorporated into EVs seem to be the key regulators of tumor-supporting processes, operating in a highly efficient manner by directly targeting mRNAs in recipient cells. In our previous study, we proved in vitro that EVs derived from invasive cells reflect a part of the specific miRNA signature of these cells compared to EVs derived from noninvasive cells, suggesting that some of these miRNAs are involved in the activation of a specific stromal microenvironment. EVs are secreted into the systemic circulation. miRNAs inside the EVs are suitable diagnostic and prognostic biomarkers because they are very stable against RNase degradation (Koga et al. 2011). Several studies have shown that EV miRNAs from urine and blood can be used to identify bladder cancer patients (Adam et al. 2013; Jiang et al. 2015; Matsuzaki et al. 2017; Wang et al. 2012). However, no published data are available focusing on the analysis of urinary EVs for discrimination between NMIBC and MIBC.

Based on our previous work (Baumgart et al. 2017b), the aim of the present study was to define a specific differential miRNA expression profile between primary tumor tissues of MIBC and NMIBC. Furthermore, the potential of miRNAs to be noninvasive diagnostic biomarkers for MIBC was evaluated using urinary EVs.

Methods

Patient material

Primary formalin-fixed, paraffin-embedded (FFPE) tumors ($n = 66$) and urine supernatants ($n = 37$; 2012–2017) stored at − 80 °C were obtained from patients undergoing transurethral bladder resection or radical cystectomy in our department. The tissue and urine samples were not paired. Urine samples were obtained before transurethral resection (TUR). The study was approved by the Saarland Ethics Committee, and informed consent was provided by all patients. Tumor stage and grade were determined according to the WHO 2004 criteria and TNM 2010 classification (Eble et al. 2004; Edge et al. 2010). Tumors were categorized into MIBC (≥ pT2) and NMIBC (pTa; pT1).

EV purification

Urinary EVs were isolated using a protocol adapted from the Total Exosome Isolation From Urine Kit (Life Technologies), as previously described (Baumgart et al. 2017b). Briefly, urine supernatant (2 ml) was centrifuged at 2000×*g* and 4 °C for 20 min and at 15,000×*g* for 30 min. The supernatant was mixed with reagent at a ratio of 1:1. The following steps were performed according to the manufacturer's

protocol. The pellet was dissolved in PBS or 2% paraformaldehyde (PFA) or was directly lysed.

Western blot analyses

EV lysates were prepared in lysis buffer [50 mM Tris; 150 mM NaCl; 1% Triton X-100 (v/v); 1% CHAPS (v/v); 0.1% SDS (m/v)] containing Protease Inhibitor Mix M (1:100; Serva). Western blotting was performed with modifications as previously published (Baumgart et al. 2017b). Immunoblotting of EV proteins was carried out overnight at 4 °C using antibodies against CD63 (1:1000; Abcam), syntenin (1:1000; Abcam) and GM130 (1:1000; Cell Signaling). Incubation within horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse IgG (1:1000) and anti-rabbit IgG (1:1000); Cell Signaling) was performed for 1 h at room temperature after washing in TBS-T. Immunoreactive bands were visualized using Immuno Blue HRP substrate (NH DyeAGNOSTICS GmbH) and detected with the Chemostar Imager system of the Intas ECL Chemocam Imager (Intas Science Imaging).

Nanoparticle tracking analysis

Particle sizes and concentrations were analyzed in a NanoSight LM10 system with NTA software version 2.3 (Malvern Panalytical GmbH) according to a previous publication (Baumgart et al. 2017a). The particle size was calculated as the median ± standard deviation of ten urinary EV samples.

Electron microscopy

EVs were fixed with 2% PFA-PBS and placed on a Formvar/carbon-coated EM grid for 30 min. The grid was washed in Aqua Dest and fixed again for 5 min with 1% glutaraldehyde. After washing in Aqua Dest, the grid was incubated in uranyl-oxalate solution (pH 7), washed again in Aqua Dest and dried. Transmission electron microscopy (Carl Zeiss GmbH) was performed at 100 kV, and electron micrographs were acquired.

Isolation of total RNA

Total RNA was isolated from FFPE samples using miRNeasy FFPE Kit (Qiagen). Twenty sections were prepared on microscope slides for macrodissection of tumor areas. Tumor sections were transferred into a reaction tube, and total RNA was isolated according to the manufacturer's protocol. Total RNA was isolated from urinary EVs using the miRNeasy Mini Kit (Qiagen). After 30 min of lysis, total RNA was extracted according to the manufacturer's protocol. The RNA concentration was measured by a NanoDrop 1000 (PeqLab Biotechnology GmbH).

miRNA expression analysis

miRNA expression analyses were performed using a human miRNA microarray (Agilent Technologies; vers. 16;) according to a previously described protocol (Baumgart et al. 2017b). After extraction of raw data using Feature Extraction software (Agilent Technologies), the data were analyzed using Qlucore (vers. 3; Qlucore). The total gene signal was normalized to the 75th percentile of signal intensity.

To validate the microarray results, qPCR was performed. Total RNA (FFPE = 100 ng; urinary EV = 10 ng) was reverse transcribed using specific primers (miR-138-5p; -144-5p; -146b-5p; -155-5p; -200a-3p; RNU48) and a TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's protocol. cDNA transcribed from urinary EVs was preamplified with TaqMan PreAmp Master Mix (Applied Biosystems) and TaqMan MicroRNA Assays (Applied Biosystems). The PreAmp product was diluted 1:5 in TE buffer (Applied Biosystems). qPCR was performed in triplicate using specific TaqMan primers and TaqMan Gene expression Master Mix (Life Technologies). The average was calculated, and the expression values of the tissue samples were normalized to those of RNU48. The expression of miRNAs from urinary EVs was normalized to the quantity of RNA used for qPCR because no suitable normalization methods for EV-derived miRNA are available.

Statistical analyses

Microarray data were statistically evaluated using Student`s t test and unsupervised unpaired hierarchical clustering using Qlucore Omics explorer (version 3; Qlucore). The parameters were set to FC > 1.5, $P < 0.05$ and variance (σ) = 0.2. The false discovery rate (FDR) was defined by using the Benjamini–Hochberg method and q was adjusted at < 0.25. The nonparametric Mann–Whitney U test (IBM SPSS Statistics 20 (IBM)) was used to analyze raw microarray data. qPCR data were analyzed using REST 2009 (vers. 2009; Technical University Munich; Qiagen) and the nonparametric Mann–Whitney U test or Kruskal–Wallis test with post hoc analysis (IBM SPSS Statistics 25 (IBM)).

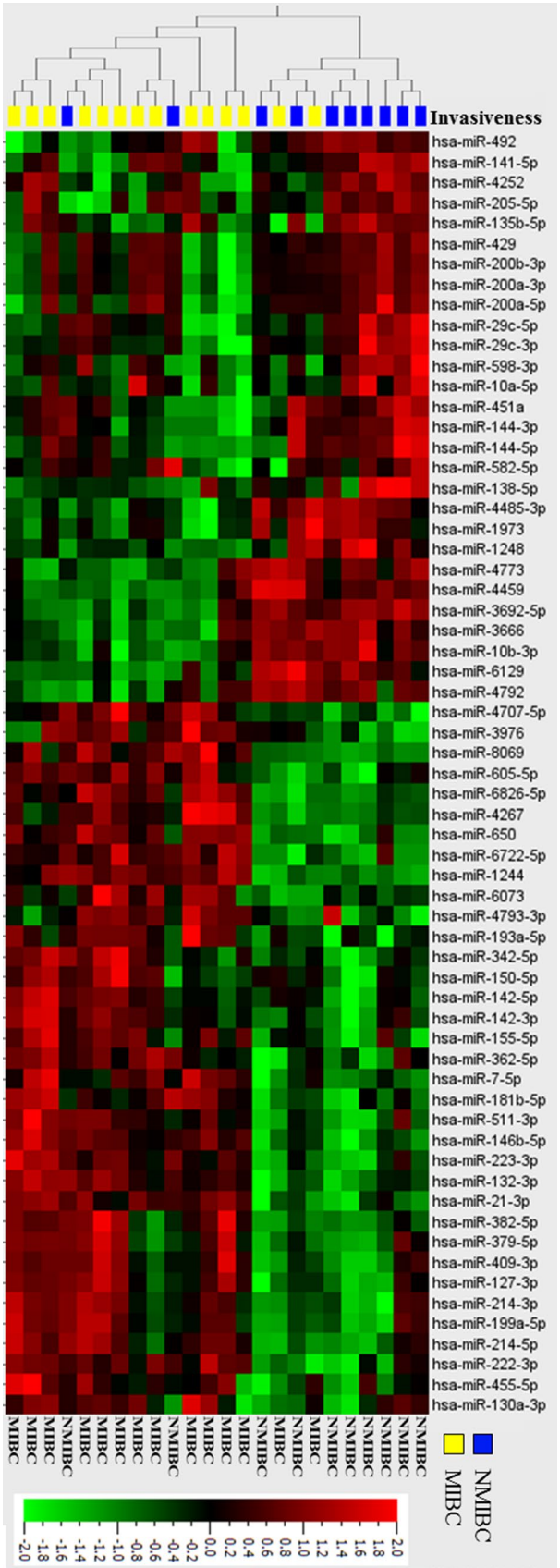

Fig. 1 Microarray analysis of miRNA expression in tumor tissues. miRNA expression analysis of muscle-invasive bladder cancer (MIBC; yellow; $n = 14$) and non-muscle-invasive bladder cancer (NMIBC; blue; $n = 10$) tissues by unsupervised hierarchical clustering analysis. The P value was determined using the Mann–Whitney U test. (P value < 0.05; fold change > 1.5; $\sigma = 0.2$)

Results

Muscle-invasive bladder cancer is characterized by a specific miRNA expression pattern

The miRNA microarray analysis (NMIBC = 10; MIBC = 14) revealed 63 miRNAs that were differentially expressed ($P < 0.05$, $q = 0.23$) between MIBC and NMIBC (Fig. 1; Table 1). In MIBC, a higher expression of 35 miRNAs as well as a lower expression of 28 miRNAs was found.

The extended validation cohort included 66 patients. Ten samples were excluded from the analysis due to insufficient amplification. In total, the miRNA expression of 56 samples (NMIBC = 22; MIBC = 36) could be analyzed by using qPCR (Table 2).

We selected two upregulated (miR-146b-5p; miR-155-5p) and three downregulated (miR-138-5p; miR-144-5p; miR-200a-3p) miRNAs for validation by qPCR based on statistical significance, fold change value and described biological function (Fig. 2). miR-138-5p (FC = 0.263), miR-144-5p (FC = 0.732) and miR-200a-3p (FC = 0.358) were confirmed to exhibit significantly lower expression in MIBC than in NMIBC ($P < 0.001$). A significantly higher expression of miR-146b-5p (FC = 7.557) and miR-155-5p (FC = 3.601) in MIBC was also demonstrated ($P < 0.001$).

Subsequently, the association of miRNA expression with T-category was analyzed in more detail (Fig. 2). In general, a significant correlation was confirmed for all miRNAs except miR-144-5p. miR-155-5p expression was significantly higher in pTa than in pT2 (FC = 3.85) and pT3–4 (FC = 3.53) ($P < 0.05$) tumors by post hoc analysis. pT2 (pTa vs pT2: FC = 5.85; pT1 vs pT2: FC = 7.28) and pT3–4 tumors (pTa vs pT3–4: FC = 8.09; pT1 vs pT3–4: FC = 10.04) exhibited significantly higher expression of miR-146b-5p than pTa and pT1 tumors ($P < 0.05$). Based on the significantly lower expression of miR-138-5p, pT3–4 (FC = 0.163) tumors can be distinguished from pTa tumors ($P < 0.05$). pT3–4 tumors significantly differed from pTa tumors based on their lower expression of miR-200a-3p (FC = 0.324; $P = 0.01$).

pT1 tumors exhibited lower expression of miR-138-5p and miR-144-5p than pTa tumors, although the difference was not significant. In contrast, miR-146b-5p, miR-155-5p and miR-200a-5p expression in pT1 tumors did not differ from that in pTa tumors.

The expression of four miRNAs was significantly associated with higher tumor grade ($P < 0.05$). Higher

Table 1 Differentially expressed miRNAs between muscle-invasive bladder cancer and non-muscle-invasive bladder cancer (microarray analysis)

miRNA	*P* value	Fold change	Expression
hsa-miR-135b-5p	0.004	0.168	Down
hsa-miR-429	0.022	0.224	Down
hsa-miR-200a-3p	0.031	0.231	Down
hsa-miR-200b-3p	0.033	0.27	Down
hsa-miR-138-5p	0.006	0.282	Down
hsa-miR-144-5p	0.005	0.316	Down
hsa-miR-144-3p	0.004	0.331	Down
hsa-miR-29c-5p	< 0.001	0.372	Down
hsa-miR-582-5p	0.011	0.374	Down
hsa-miR-10a-5p	0.016	0.375	Down
hsa-miR-598-3p	0.031	0.376	Down
hsa-miR-451a	0.021	0.409	Down
hsa-miR-29c-3p	< 0.001	0.412	Down
hsa-miR-141-5p	0.019	0.428	Down
hsa-miR-200a-5p	0.021	0.44	Down
hsa-miR-4485-3p	0.01	0.472	Down
hsa-miR-6129	0.048	0.484	Down
hsa-miR-1248	0.048	0.489	Down
hsa-miR-3692-5p	0.016	0.491	Down
hsa-miR-4252	0.023	0.497	Down
hsa-miR-4773	0.013	0.505	Down
hsa-miR-3666	0.036	0.507	Down
hsa-miR-4792	0.038	0.523	Down
hsa-miR-205-5p	0.036	0.525	Down
hsa-miR-10b-3p	0.013	0.533	Down
hsa-miR-1973	0.02	0.533	Down
hsa-miR-4459	0.028	0.55	Down
hsa-miR-492	0.033	0.573	Down
hsa-miR-362-5p	0.042	1.742	Up
hsa-miR-6073	0.048	1.786	Up
hsa-miR-4793-3p	0.032	1.824	Up
hsa-miR-130a-3p	0.019	1.842	Up
hsa-miR-3976	0.016	1.857	Up
hsa-miR-8069	0.006	1.958	Up
hsa-miR-605-5p	0.013	1.994	Up
hsa-miR-181b-5p	0.007	2.063	Up
hsa-miR-6722-5p	0.003	2.191	Up
hsa-miR-142-3p	0.011	2.289	Up
hsa-miR-4707-5p	0.008	2.294	Up
hsa-miR-199a-5p	0.037	2.353	Up
hsa-miR-4267	0.003	2.358	Up
hsa-miR-193a-5p	0.001	2.370	Up
hsa-miR-132-3p	0.019	2.383	Up
hsa-miR-455-5p	0.02	2.406	Up
hsa-miR-6826-5p	< 0.001	2.438	Up
hsa-miR-379-5p	0.042	2.499	Up
hsa-miR-342-5p	0.003	2.500	Up
hsa-miR-409-3p	0.015	2.531	Up
hsa-miR-142-5p	0.003	2.545	Up
hsa-miR-155-5p	< 0.001	2.571	Up
hsa-miR-214-3p	0.031	2.729	Up
hsa-miR-222-3p	0.011	2.779	Up
hsa-miR-511-3p	0.006	2.857	Up
hsa-miR-214-5p	0.036	2.876	Up
hsa-miR-382-5p	0.031	2.908	Up
hsa-miR-650	< 0.001	2.924	Up
hsa-miR-223-3p	0.042	2.933	Up
hsa-miR-1244	0.013	3.080	Up
hsa-miR-127-3p	0.011	3.129	Up
hsa-miR-150-5p	0.003	3.403	Up
hsa-miR-21-3p	0.004	3.428	Up
hsa-miR-146b-5p	0.001	3.904	Up
hsa-miR-7-5p	0.001	4.605	Up

Table 2 Clinicopathological parameters of the study cohorts

	Tumor tissue ($n=56$)	Urinary EV ($n=37$)
Age (years)		
Mean/median (range)	68/68 (30–87)	68/70 (43–84)
Sex		
Male	40 (71.4%)	27 (73.0%)
Female	16 (28.6%)	10 (27.0%)
T		
pTa	28 (50.0%)	13 (35.2%)
pT1	6 (10.7%)	4 (10.8%)
pT2	7 (12.5%)	9 (24.3%)
pT3/T4	15 (26.8%)	11 (29.7%)
N		
pN0	12 (21.4%)	8 (21.6%)
pN+	10 (17.9%)	7 (18.9%)
NX	34 (60.7%)	22 (59.5%)
Grade		
G1	7 (12.5%)	1 (2.7%)
G2	25 (44.6%)	12 (32.4%)
G3	24 (42.9%)	24 (64.9%)

EV extracellular vesicles

expression of miR-146b-5p (G1 vs. G3: FC = 4.17; G2 vs. G3: FC = 4.97) and lower expression of miR-138-5p (G1 vs. G3: FC = 0.275; G2 vs. G3: FC = 0.278) were present in G3 tumors than in either G1 or G2 tumors ($P < 0.05$; Fig. 3). miR-155-5p expression was upregulated in G3 tumors relative to that in G2 tumors (FC = 3.22; $P = 0.001$). MiR-200a-3p was found to be downregulated in G3 vs G2 tumors (FC = 0.411; $P = 0.009$). The expression of miR-144-5p was not significantly deregulated

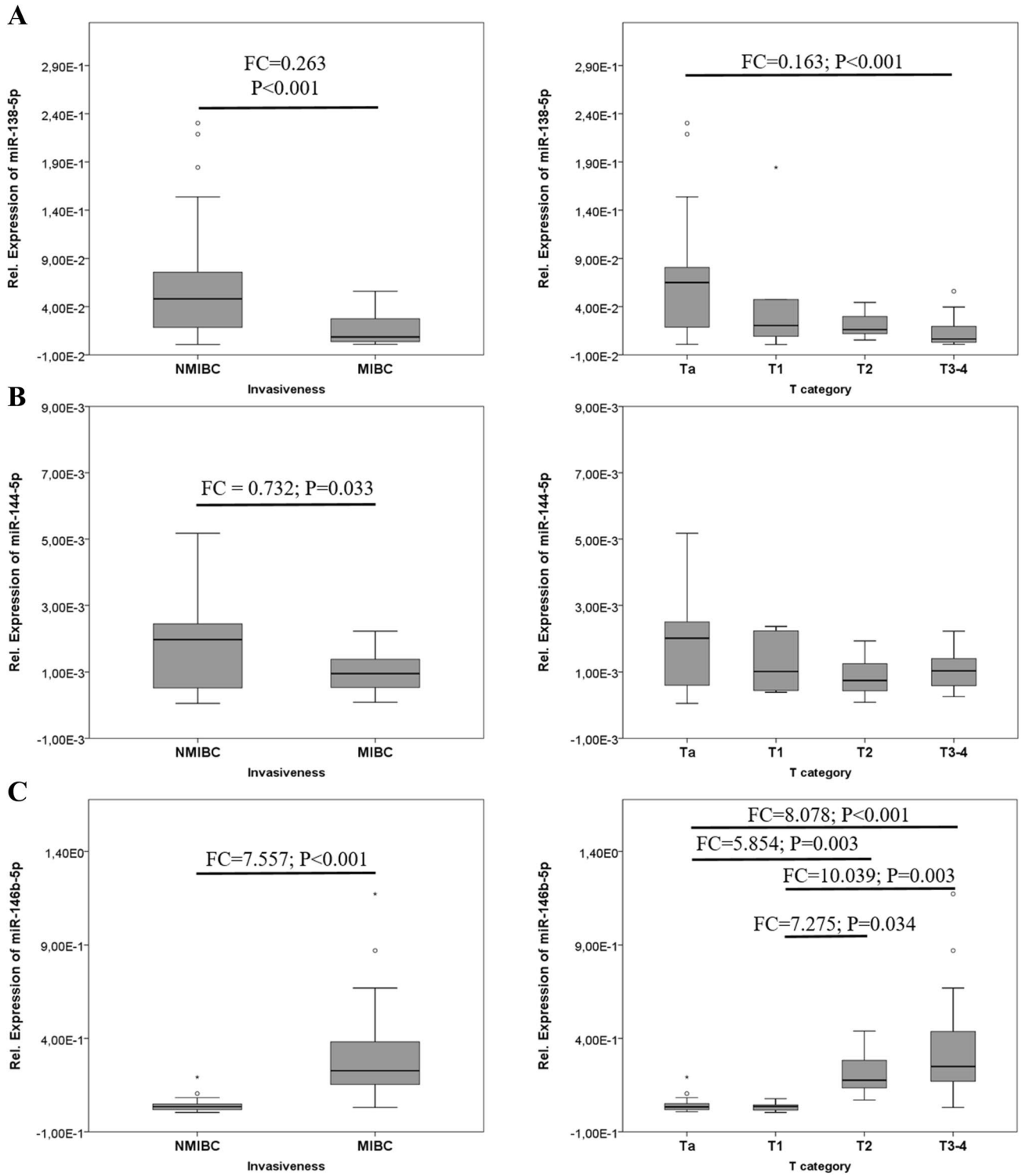

Fig. 2 Validation of specific deregulated miRNAs in muscle-invasive bladder cancer by RT-PCR. The relative expression levels of miR-138-5p (**a**), miR-144-5p (**b**), miR-146b-5p (**c**), miR-155-5p (**d**) and miR-200a-3p (**e**) in MIBC ($n=22$) compared to NMIBC ($n=34$). Furthermore, the relative expression levels of all miRNAs were analyzed depending on the T-category (pTa: $n=28$; pT1: $n=6$; pT2: $n=7$; pT3–4: $n=15$). Expression was normalized to that of the reference gene RNU48. *P* values were determined by using the Mann–Whitney *U* test or the Kruskal–Wallis test including post hoc analysis, and the fold change (FC) was calculated with REST software

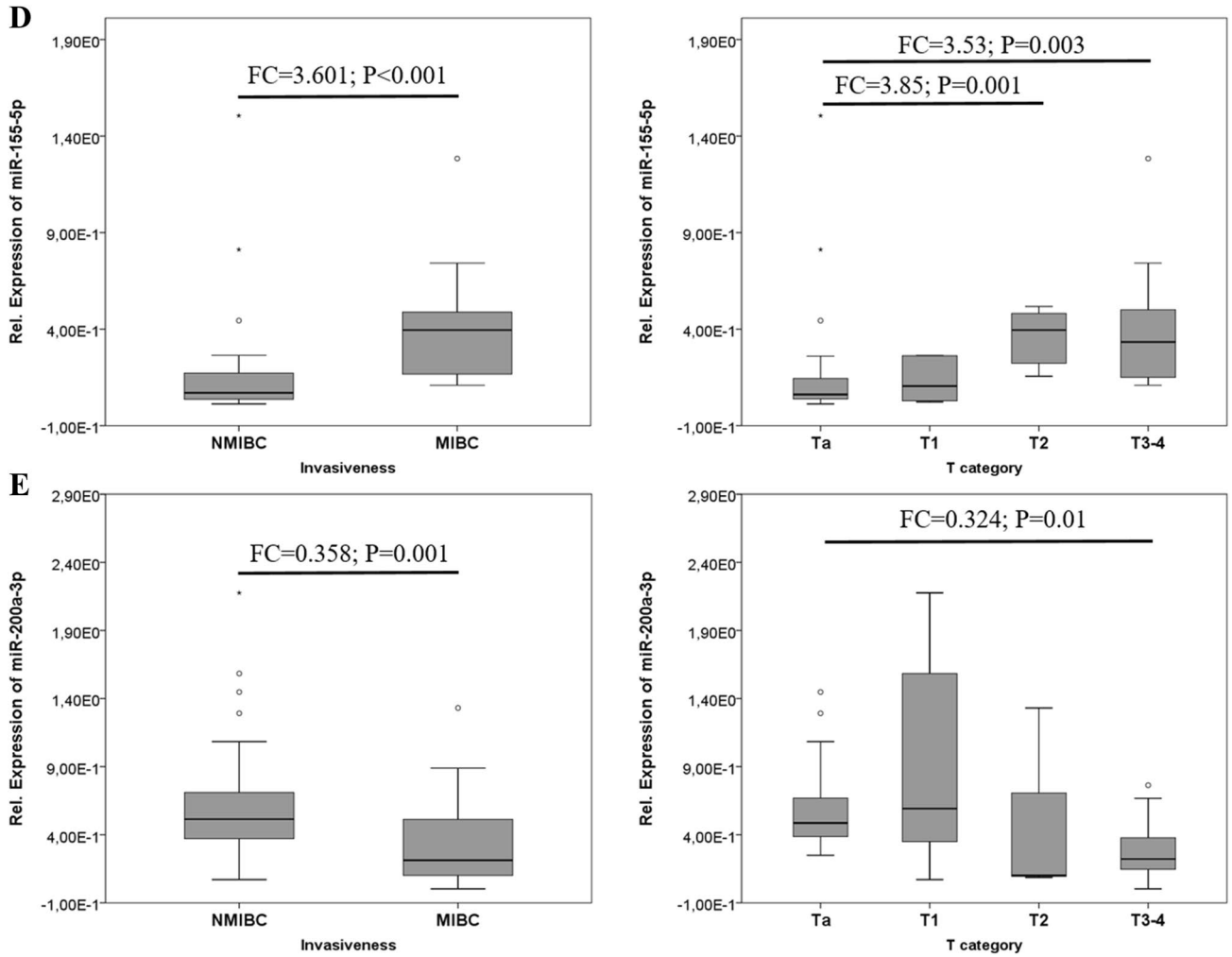

Fig. 2 (continued)

depending on tumor grade. The qPCR data for all five miRNAs revealed no significant expression differences between N0 and N + tumors ($P > 0.05$).

Extracellular vesicles are secreted into the urine of bladder cancer patients

Isolated EVs contained high amounts of the EV markers CD63 and syntenin without cellular contamination, as demonstrated by the absence of the protein GM130 (Fig. 4b). Electron microscopy of two independent urinary EV samples verified the presence of rounded membrane-bound vesicles with a size of 48 nm (SD ± 24 nm; Fig. 4b). Particle size measured using nanoparticle tracking analysis (NTA) revealed a median EV size of ~ 125 nm ± 35 nm (SD).

miRNAs are differentially expressed in EVs isolated from the urine of bladder cancer patients

The clinical relevance of miRNA expression in primary tumors as a potential diagnostic marker was further investigated in urinary EVs. Differential expression could be verified for two miRNAs in urinary EVs from 37 patients (Fig. 5). The expression levels of miR-146b-5p (FC = 7.84; $P = 0.004$) and miR-155-5p (FC = 5.24; $P = 0.036$) were significantly higher in urinary EVs from MIBC patients than in those from NMIBC patients. A significant association with T-category was confirmed by the Kruskal–Wallis test. The expression of both miRNAs was significantly upregulated in pT2 tumors relative to that in pTa tumors, but did not differ between pTa and pT3–4 tumors. The expression of these miRNAs in pT1 EVs was similar to that in pTa EVs. The expression differences between pT1 and pT2 tumors were

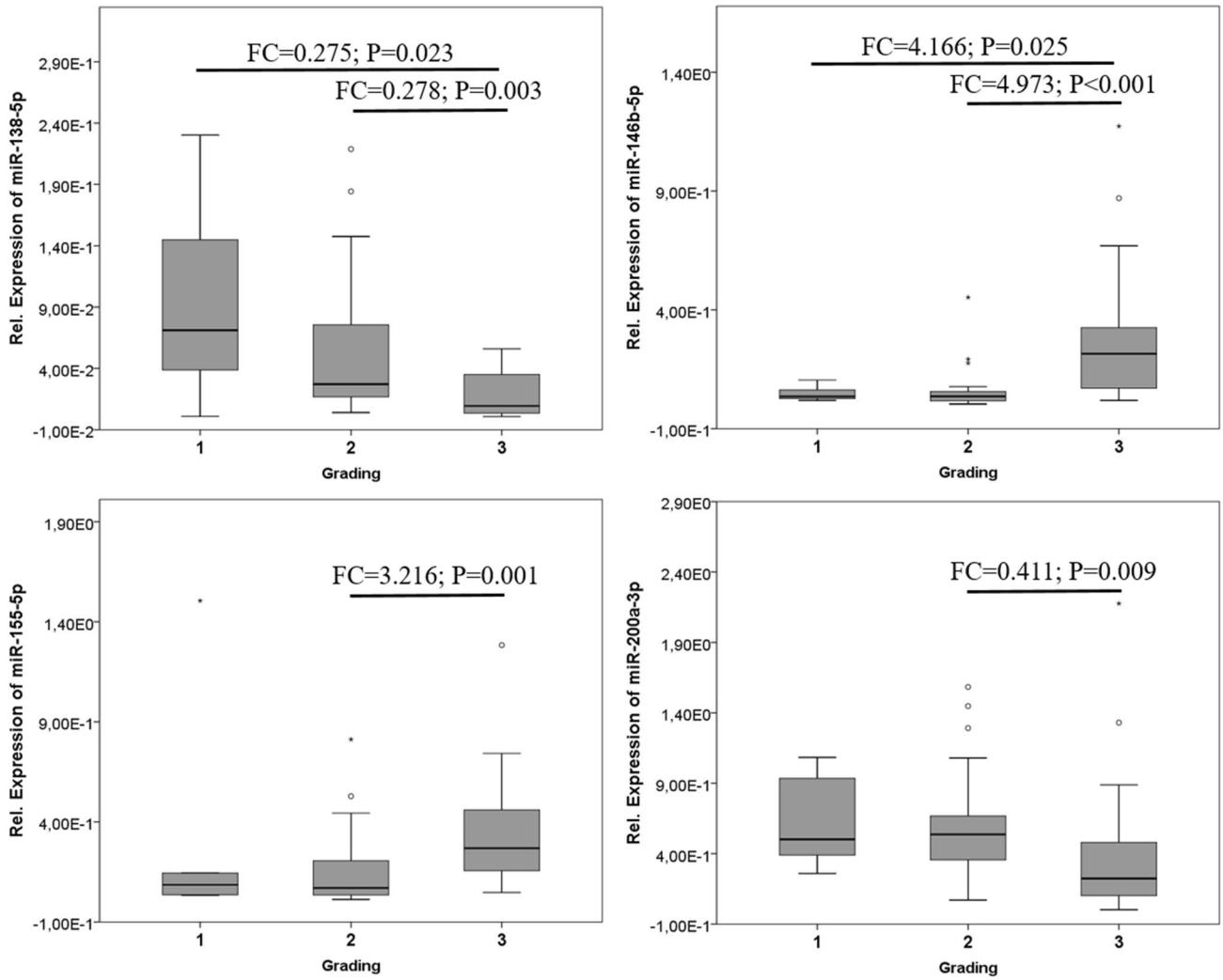

Fig. 3 Validation of the miRNA expression of specific deregulated miRNAs depending on the tumor grade. The relative expression levels of miR-138-5p, miR-146b-5p, miR-155-5p and miR-200a-3p depending on the tumor grade (G1: $n=7$; G2: $n=25$; G3: $n=24$). Expression was normalized to that of the reference gene RNU48. *P* values were determined using the Kruskal–Wallis test including post hoc analysis, and the fold change (FC) was calculated using REST software

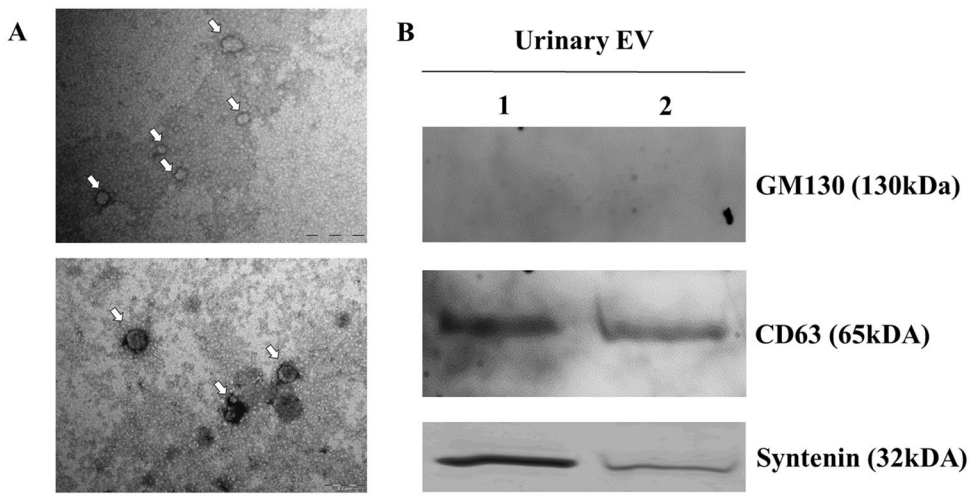

Fig. 4 Extracellular vesicles isolated from the urine of bladder cancer patients. **a** Extracellular vesicles (EVs) were obtained from the urine of bladder cancer patients and analyzed by electron microscopy. (Scale 200 nm). **b** Western blot analyses of two different urinary EV samples for the exosomal markers CD63 (65 kDa) and syntenin (32 kDa) and the cellular contamination marker GM130 (130 kDa)

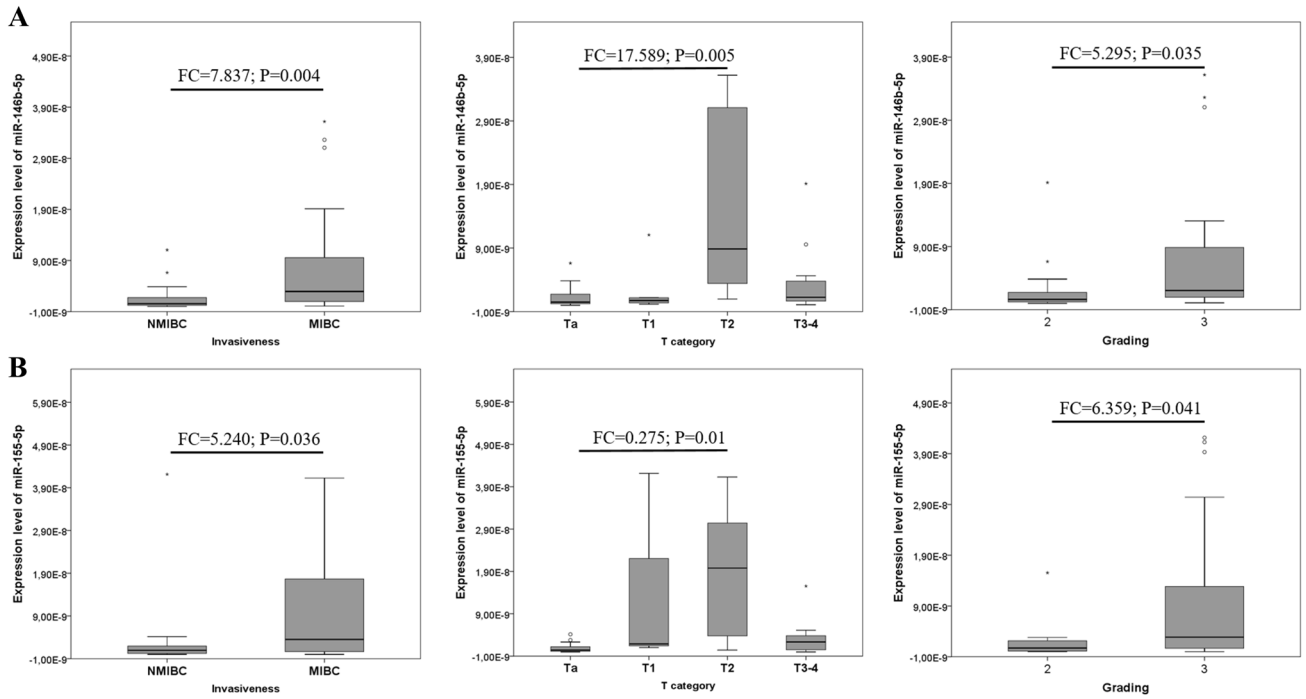

Fig. 5 Validation of the miRNA expression of selected miRNAs in EVs isolated from urine. Expression levels of miR-146b-5p (**a**) and miR-155-5 (**b**) in MIBC ($n=20$) and NMIBC ($n=17$) In addition, the expression levels of both miRNAs were analyzed by T stage (pTa: $n=13$; pT1: $n=7$; pT2: $n=9$; pT3–4: $n=11$) and grade (G2: $n=12$; G3: $n=24$). The expression was not normalized. *P* values were determined using the Mann–Whitney *U* test or the Kruskal–Wallis test including post hoc analysis, and the fold change (FC) was calculated with REST software

nonsignificant. In most cases, miR-144-5p was not detectable in urinary EVs.

Furthermore, a significantly higher expression of miR-146b-5p (FC = 5.30; $P=0.035$) and miR-155-5p (FC = 6.36; $P=0.041$) was revealed in urinary EVs of G3 tumors than in G2 tumors. No correlation between lymph node status and miRNA expression was found for urinary EVs ($P>0.05$).

Discussion

The molecular pathway of MIBC development is different from that of NMIBC, and specific genetic alterations, including mutations of the tumor suppressor genes *p53* and *PTEN*, can be defined (Robertson et al. 2017). Several studies have demonstrated that miRNAs are also involved in the tumorigenesis of bladder cancer, but knowledge about their specific role in the development of an invasive phenotype is still limited. Our previous in vitro study revealed an invasion-associated miRNA signature in both tumor cell lines and their secreted EVs (Baumgart et al. 2017a). Consequently, we hypothesized that MIBC is characterized by a specific miRNA expression pattern in primary tumors as well as in urinary EVs.

For the first time, we describe that MIBC is characterized not only by a specific invasion-associated miRNA pattern in primary tumors, but also by certain deregulated miRNAs in urinary EVs. Our earlier in vitro study confirmed six miRNAs (including miR-200a-3p) as significantly differentially expressed in both invasive cell lines as well as MIBC (Baumgart et al. 2017b). Some of the miRNAs identified have already been published as general biomarkers for MIBC (Dyrskjot et al. 2009; Pignot et al. 2013). MiR-200a-3p, a member of the miR-200 family, is frequently described as an important regulator of EMT and therefore of an invasion-associated phenotype (Dyrskjot et al. 2009; Pignot et al. 2013). By overexpression of miR-200a-3p in T24 cells, we confirmed the role of this miRNA in tumor-promoting processes, such as proliferation, migration and invasion (data not shown). Other miRNAs identified in our study have been found to be involved in the tumorigenesis of bladder cancer and other cancers (Gao et al. 2016; Song et al. 2010; Yang et al. 2016).

After confirming significant miRNA expression differences between MIBC and NMIBC in general, we further analyzed the miRNA expression between T-categories in more detail. Whereas a significant expression difference between pTa and ≥ pT2 tumors was found for four out of the five miRNAs, only one miRNA displayed significant

differences between pT1 and muscle-invasive T-categories. Another two miRNAs (miR-138-5p and miR-144-5p) were found to have similar expression levels in pT1 and MIBC tumors, but different expression in pTa tumors. For miR-138-5p, an even further downregulation between pT2 and pT3–4 tumors was observed, indicating that low expression correlates with an aggressive phenotype. As shown in earlier studies, miR-138-5p expression is significantly downregulated in bladder cancer relative to that in normal tissue (Sun et al. 2015; Yang et al. 2016). Low expression of this miRNA results in higher expression of the oncogene *survivin* and the EMT-associated protein ZEB2. Therefore, miR-138-5p seems to act as an important tumor suppressor (Sun et al. 2015; Yang et al. 2016). In contrast, both pTa and pT1 tumors were segregated from ≥ pT2 tumors by significant overexpression of two miRNAs (miR-146b-5p and miR-155-5p), suggesting a direct association with muscle invasiveness. To date, nothing is known about the role of these miRNAs in the development of an invasive phenotype in bladder cancer. MiR-146b-5p expression is upregulated in bladder cancer tissue relative to that in the normal bladder mucosa. The function of this miRNA, however, is unknown (Song et al. 2010). In thyroid cancer, miR-146b-5p overexpression is associated with invasion and advanced tumor stage via the stimulation of EMT-associated processes (Deng et al. 2015). For the first time, we demonstrate deregulation of miR-155-5p expression in MIBC compared to that in NMIBC. This miRNA is also upregulated in clear cell renal cell carcinoma (ccRCC) and affects the proliferation and invasion of ccRCC cells by targeting *E2F2*, a key player in apoptosis, proliferation and several tumor-promoting processes in vivo and in vitro (Gao et al. 2016). These data support the role of miR-155-5p in the development of an invasive phenotype. Our results suggest that some miRNAs characterize the potential for invasion into the lamina propria, whereas others define the ability to further invade the muscle layer.

We confirmed a distinct miRNA pattern in MIBC and high-grade tumors, suggesting a role for predicting an invasive phenotype before it can be observed by histopathological examination. Of particular interest in clinical practice is the risk of pT1 high-grade tumor progression as a potential indication for early cystectomy. Further studies must prove the additional impact of miRNA biomarkers for decision making in pT1G3 tumors, which were excluded from the present study, to obtain a more homogenous population of NMIBC cases.

In addition to tissue samples, liquid biopsies represent a technical alternative for identifying biomarkers that could help to overcome sampling errors associated with intratumoral heterogeneity. Until now, urine cytology represents the gold standard for bladder cancer diagnosis. High-grade invasive tumors can be detected with high accuracy. However, an accurate prediction of the invasive potential is limited. As tumor cells release many molecules into the circulation, the analysis of nucleic acids and proteins in blood and urine (e.g., cell-free miRNA, DNA, and proteins) should result in a more representative picture of molecular complexity and heterogeneity. Therefore, we assessed the suitability of invasion-associated miRNAs in urinary EVs of bladder cancer patients as potential diagnostic biomarkers. In general, miRNA expression studies (including those on miR-21-5p; miR-100; miR-146b-5p; and the miR-200 family) indicate that liquid biopsies (e.g., urine, plasma, serum) are suitable for discriminating patients with bladder cancer from healthy individuals (Matsuzaki et al. 2017; Song et al. 2010; Yang et al. 2016). Nevertheless, no data have been published focusing on discrimination between MIBC and NMIBC using miRNAs in EVs, particularly those in the urine. We verified two out of five miRNAs (miR-146b-5p and miR-155-5p) underlying the potential of EV miRNAs as potential noninvasive diagnostic markers of MIBC.

In general, the heterogeneity of urinary EVs still represents a problem for the development of robust diagnostic tools. Urine contains not only exclusively tumor-associated EVs, but also EVs secreted by other cell types (e.g., endothelial cells, fibroblasts, normal urothelium) of the lower and upper urinary tract. Although tumor EVs probably represent the largest proportion of urinary EVs, other EVs could dilute the tumor-specific miRNA expression pattern. Further studies should investigate if microarray screening starting directly from urinary EV miRNAs instead of translating results from tumor tissue analysis could increase diagnostic accuracy. In addition, the lack of significant differences between MIBC and NMIBC for some miRNAs may be caused in part by an aberrant expression pattern in patients with pT3–4 tumors. Based on the miRNA expression in urinary EVs, pTa tumors could be clearly differentiated from pT2 tumors but not from pT3–4 tumors, despite significant differences in tumor tissue miRNAs. Necrosis is often present in pT3–4 tumors, potentially reducing the contact surface of vital tumor cells with urine and thus leading to a decreased tumor EV concentration. Urine sediment is also used for miRNA analysis. However, quantification is again influenced by the number of shed tumor cells in the urine. Because EVs protect miRNAs from degradation EVs seems to be a better source for miRNA analysis than "free" miRNA, although data are missing comparing both variants in bladder cancer.

Further studies are needed to optimize the normalization of miRNA expression as well as to overcome the heterogeneity issue inherent to urinary EVs by the enrichment of tumor-associated EVs. Nevertheless, our data indicate that miRNAs in urinary EVs are potential markers to identify MIBC based on a noninvasive test. Whether urine diagnostics based on EV miRNA expression can facilitate differential therapy

without diagnostic transurethral resection must be investigated in further studies.

Our study is limited by its retrospective nature based on a selected test cohort. Furthermore, analysis of miRNA expression in MIBC was predominantly performed on cystectomy samples. Further investigations need to prove that TUR samples are equally suitable for precise miRNA-based classification of invasiveness. We investigated each candidate miRNA separately. After confirming the relevance of these miRNAs in independent cohorts, miRNA signatures must be developed for discriminating between MIBC and NMIBC tumors. Urinary samples from grade 1 tumor patients are underrepresented compared to tissue analyses. In addition to the discussed problems in EV miRNA analysis, this could cause distinct results in urine compared tissue samples. Influence of tumor grade on miRNA expression in MIBC compared to NMIBC has to be investigated by statistics in larger cohorts.

Conclusions

miRNAs from tissue samples as well as from urinary EVs are promising biomarkers to identify bladder carcinomas with an invasive phenotype and could therefore support decision making for or against aggressive therapies. Whether the invasive potential can be identified on the basis of the urinary EV miRNA profile alone seems worthy of further investigation.

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Compliance with ethical standards

Conflict of interest The 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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