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RESEARCH ARTICLE

MicroRNA expression profiles in muscle-invasive bladder cancer: identification of a four-microRNA signature associated with patient survival

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Abstract Bladder cancer ranks the second most common genitourinary tract cancer, and muscle-invasive bladder cancer (MIBC) accounts for approximately 25 % of all bladder cancer cases with high mortality. In the current study, with a total of 202 treatment-naïve primary MIBC patients identified from The Cancer Genome Atlas dataset, we comprehensively analyzed the genome-wide microRNA (miRNA) expression profiles in MIBC, with the aim to investigate the relationship of miRNA expression with the progression and prognosis of MIBC, and generate a miRNA signature of prognostic capabilities. In the progression-related miRNA profiles, a total of 47, 16, 3, and 84 miRNAs were selected for pathologic T, N, M, and histologic grade, respectively. Of the eight most important progression-related miRNAs, four (let-7c, mir-125b-1, mir-193a, and mir-99a) were significantly associated with survival of patients with MIBC. Finally, a four-miRNA signature was generated and proven as a promising prognostic parameter. In summary, this study identified the specific miRNAs associated with the progression and aggressiveness of MIBC and a four-miRNA signature as a promising prognostic parameter of MIBC.

Keywords Muscle-invasive bladder cancer · microRNA · Biomarker · Prognosis

Introduction

Bladder cancer (BC) is the second most prevalent genitourinary cancer in the USA, with approximately 74,000 new cases, and 16,000 BC-related deaths are projected to occur in 2015 [1]. At initial diagnosis, about 25 % of BC patients present with muscle-invasive bladder cancer (MIBC) [2], for which radical cystectomy remains the standard treatment of choice [3, 4]. However, current staging systems based on tumor-node-metastasis (TNM) classification and pathological grade are insufficient to predict patient outcome; as such, novel and efficient prognostic molecular biomarkers for MIBC should be identified [5, 6].

Non-coding RNAs are a class of endogenous RNA molecules, which regulate vital biological processes including cell division, differentiation, apoptosis, and migration [7, 8]. As a member of non-coding RNAs, microRNA (miRNA) has been extensively investigated. miRNA could modulate the expression levels of target genes by repressing translation or cleaving mRNA transcripts in a sequence-specific manner [9]. A

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growing body of evidence suggests that miRNAs are aberrantly expressed in human cancers such as lung cancer, breast cancer, BC, and renal cell carcinoma and exert important functions in the initiation, development, and progression of these cancer types [10–15]. To date, various miRNA expression profiles with prognostic values for BC patients have been identified [16–19]. However, the participants of those studies were heterogeneous with both non-muscle-invasive BC (NMIBC) and MIBC patients included, and the prognostic performance of the identified miRNA signatures needs further validation.

In the present study, with data identified from The Cancer Genome Atlas (TCGA) project [20], we explored the miRNAs associated with progression and prognosis of MIBC, with the aim to generate a miRNA signature that could predict the prognosis of MIBC patients.

Materials and methods

Study population

The study was approved by the Institutional Review Board of Nanjing First Hospital, Nanjing Medical University. From the multi-institutional TCGA database (http://cancergenome.nih.gov/) of patients undergoing transurethral resection of bladder tumor or radical cystectomy between 1999 and 2013 [20], those with pathologically diagnosed MIBC (T2–T4) were enrolled in the current study. Informed consent was obtained from all individual participants included in the TCGA project. The complete clinical and pathological data (levels 1 and 2) were downloaded and re-evaluated by two expert genitourinary pathologists (YCZ and XBY) according to 2009 TNM classification and 2004 World Health Organization criteria, as recommended by the European Association of Urology guidelines [4]. The MIBC patients that received preoperative therapy (chemotherapy or radiation therapy) were excluded. Overall, a total of 202 MIBC patients with detailed clinicopathological data including age, gender, race, TNM stage, and histological subtype and grade were identified (Table 1). The MIBC patients were routinely followed up with the end point set as overall survival (OS), which was defined as the time interval between initial surgical resection and date of death or last follow-up.

Microarray data procession

The genome-wide miRNA expression levels in MIBC tumor tissues were profiled using the Illumina HiSeq platform (Illumina Inc., San Diego, CA, USA) and presented as reads per million counts (RPM). After downloaded from TCGA data portal (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp), the summary miRNA expression data was processed with BRB-Array Tools (version 4.4.0; National Cancer Institute, Bethesda, MD, USA) which were developed by the BRB-Array Tools Development Team (http://brb.nci.nih.gov/BRB-ArrayTools.html) [21]. According to the following predesigned selection criteria: (1) more than 1 RPM in at least 10 % of all samples and (2) with changes of more than 1.5-fold from the median value in at least 20 % of samples, the eligible miRNAs were retained and log2 transformed for further analysis.

Table 1 Clinicopathological characteristics of patients with muscle-invasive bladder cancer

	MIBC patients (n=202)
Age, years	
Median (IQR)	68 (60, 76)
Gender, *n* (%)	
Female	52 (25.7 %)
Male	150 (74.3 %)
Race, *n* (%)	
Caucasian	169 (83.7 %)
African	12 (5.9 %)
Asian	21 (10.4 %)
Histologic subtype, *n* (%)	
Non-papillary	142 (70.3 %)
Papillary	60 (29.7 %)
Histologic grade, *n* (%)	
High grade	192 (95.0 %)
Low grade	10 (5.0 %)
Pathologic stage, *n* (%)	
Stage II	68 (33.7 %)
Stage III	71 (35.1 %)
Stage IV	63 (31.2 %)
Pathologic T, *n* (%)	
T2	76 (37.6 %)
T3	99 (49.0 %)
T4	27 (13.4 %)
Pathologic N, *n* (%)	
N0	123 (60.9 %)
N1–3	62 (30.7 %)
NX	17 (8.4 %)
Pathologic M, *n* (%)	
M0	100 (49.5 %)
M1	4 (2.0 %)
MX	98 (48.5 %)

MIBC muscle-invasive bladder cancer, *IQR* inter-quartile range, *NX* regional lymph node unknown, *MX* metastasis status unknown

Statistical analysis

Continuous variables were presented as mean±standard deviation or median and inter-quartile range (IQR) according to

the different normality status determined by Kolmogorov–Smirnov and Shapiro–Wilk tests; categorical variables were expressed as counts and percentages. The miRNA expression levels between different groups (T3–4 vs. T2, N1–3 vs. N0, M1 vs. M0, and high grade vs. low grade) were ascertained with Student's *t* test embedded in BRB-Array Tools ($P<0.01$), and the unsupervised hierarchical cluster analysis was performed with Euclidian distance and average linkage methods.

To identify the miRNAs of prognostic values, the univariate Cox proportional hazard regression analysis was applied, and a risk score formula was developed by combining the expression level (**Expr**) weighted by the regression coefficient (**B**), which was calculated as follows: risk score=$\sum_{i=1}^{n}$**Expr***i** **B***i* [14, 22]. Survival curves were generated by the Kaplan–Meier method and compared using the log-rank test. The variables with missing data less than 10 % were included in the univariate Cox analyses, and those with a threshold *P* value <0.10 were further included in the multivariate Cox proportional hazard regression analysis. The results yielded by Cox regression analyses were reported as hazard ratio (HR) and the corresponding 95 % confidential interval (95 % CI). The statistical analyses were performed using BRB-Array Tools and SPSS (version 21.0; SPSS Institute Inc., Chicago, IL, USA), and statistical significance was considered as a two-sided *P* value <0.05 unless specifically indicated.

Results

Baseline characteristics of study subjects

The detailed clinical and pathological characteristics of study population were summarized in Table 1. All 202 patients enrolled in this study were clinically and pathologically diagnosed with MIBC and did not receive any neoadjuvant therapy. The median age for all patients was 68 years (IQR: 60–76 years), and male predominance was observed (male to female ratio: 2.88). A total of 40 patients died during the median follow-up interval of 6.9 months (IQR: 3.7–12.4 months).

miRNAs associated with tumor progression of MIBC

Class comparison analyses were conducted to identify miRNAs related to progression for each pathological feature. A summary of 47 miRNAs were identified for pathologic T, 16 for pathologic N, 3 for pathologic M, and 84 for histologic grade (Fig. 1 and Supplementary Table S1). As presented in Fig. 1, eight miRNAs (let-7c, mir-125b-1, mir-127, mir-193a, mir-199a-1, mir-199a-2, mir-199b, and mir-99a) were significantly correlated with the three variables (pathologic T, N, and histologic grade). In addition, the unsupervised hierarchical clustering with miRNA expression data could clearly separate different classes according to pathologic T, N, and histologic grade (Fig. 2).

Establishment of miRNA signature associated with MIBC patient survival

To identify the potential miRNAs with prognostic capabilities, the eight most important progression-related miRNAs were subjected to univariate Cox proportional hazard regression analysis. Four miRNAs (let-7c, mir-125b-1, mir-193a, and mir-99a) were significantly associated with MIBC patients' OS (Supplementary Table S2), which were further validated by the Kaplan–Meier survival curves (Fig. 3). Subsequently, a four-miRNA signature risk score was generated (Fig. 4), and the 202 MIBC patients were then divided into two groups: high risk ($n=101$) and low risk ($n=101$). As shown in the Kaplan–Meier survival curves (Fig. 4d), the high-risk patients exhibited a shorter median OS than the low-risk ones (high vs. low risk: 24.0 months vs. not reached; $P=0.034$). The differences in patients' OS corresponded to a HR of 2.021 (95 % CI: 1.042–3.921; $P=0.037$) derived from the univariate Cox regression analysis (Table 2). However, the four-miRNA signature did not pass the multivariate analysis with all significant variables such as pathologic stage, T, and N included, and only pathologic T was proven as an independent prognostic parameter (HR=2.733, 95 % CI: 1.125–6.640; $P=0.026$).

Discussion

BC is a heterogeneous entity with two distinct subtypes: NMIBC and MIBC, which poses different challenges for clinical management [3]. The identification and validation of novel molecular biomarkers for MIBC comprises an important area of clinical cancer research [5, 23]. In the present study, we explored the genome-wide miRNA expression profiles in MIBC tumor tissues and identified a four-miRNA (let-7c, mir-125b-1, mir-193a, and mir-99a) signature derived from the progression-related miRNAs, which could predict MIBC patient survival.

As an endogenous group of small, single-stranded, and non-coding RNAs, miRNAs are involved in various biological processes including cellular differentiation, apoptosis, and proliferation [8]. Emerging evidence has suggested the involvement of miRNAs in the intercellular communications through extracellular vesicles, which advanced the understanding of carcinogenic roles of miRNAs [24–26]. The aberrantly expressed miRNAs could be tracked in the initiation, progression, and metastasis of human malignancies, which suggested that miRNAs could serve as a potent group of therapeutic targets and biomarkers of diagnosis and prognosis [14, 27, 28]. In the current study, with 202 treatment-naïve primary MIBC patients strictly selected from the TCGA project, we explored the global miRNA expression profiles in MIBC

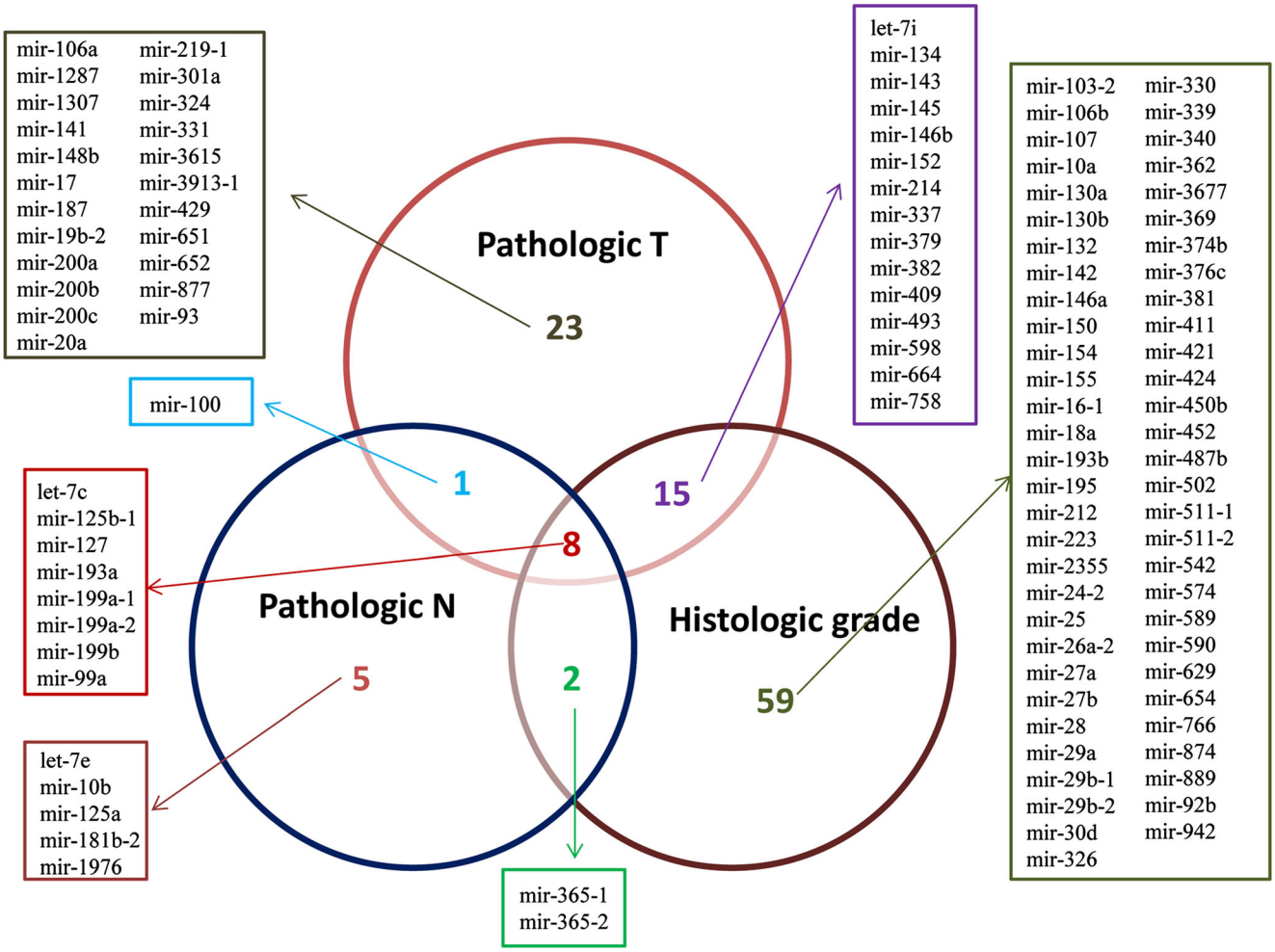

Fig. 1 MicroRNAs associated with progression of muscle-invasive bladder cancer. Venn diagram of microRNAs related to pathologic T (n=47), N (n=16), and histologic grade (n=84), which comprised the progression signature

tumor tissues. Overall, a total of 47 miRNAs were selected for pathologic T, 16 for pathologic N, 3 for pathologic M, and 84 for histologic grade. Of note, only four pathologic M1 patients were included in this study as those with distant metastasis were rarely recommended to undergo surgery, which could explain the limited number of miRNAs (n=3) differentially expressed between pathologic M1 and M0 tumor tissues. Among those significant miRNAs, eight were aberrantly expressed among different classes (T3–4 vs. T2, N1–3 vs. N0, and high grade vs. low grade) and subjected to the univariate Cox regression and Kaplan–Meier survival analyses. Of the eight miRNAs, four (let-7c, mir-125b-1, mir-193a, and mir-99a) were significantly associated with the MIBC patients' OS. Subsequently, a four-miRNA signature was generated, which could predict survival of patients with MIBC. To the best of our knowledge, this is the most comprehensive study with the largest cohort of MIBC patients (n=202) to explore the genome-wide miRNA expression profiles in MIBC tumor tissues and association of miRNA expression levels with clinical phenotypes and prognosis.

Among the four risky miRNAs, let-7c and mir-99a were organized in a cluster that resided within human chromosome 21q21.1, while mir-125b-1 and mir-193a were localized on human chromosome 11q24.1 and 17q11.2, respectively. As one member of let-7 family, let-7c has been well documented as a potent tumor suppressor [29]. The previous studies have demonstrated the reduced expression levels of let-7c in BC tumor tissues [30, 31], which were contrary to our results as the high expression levels of let-7c were significantly with a poor prognosis in MIBC patients. Even though mir-99a has been proved to be downregulated in BC tissues and inhibit the proliferation of BC cells [30, 32], its role as a tumor suppressor has been challenged [33], which was in agreement of the findings of current study. In a study with 34 BC patients, mir-125b was highly expressed in the pathologic T2–3 tumors compared with Ta tissues, which was in line with the current study [34]. Furthermore, we found that MIBC patients with higher mir-125b-1 levels exhibited shorter survival intervals, which suggested that mir-125b-1 could serve as a potent oncogene [35, 36]. miR-193a was shown to promote in vivo

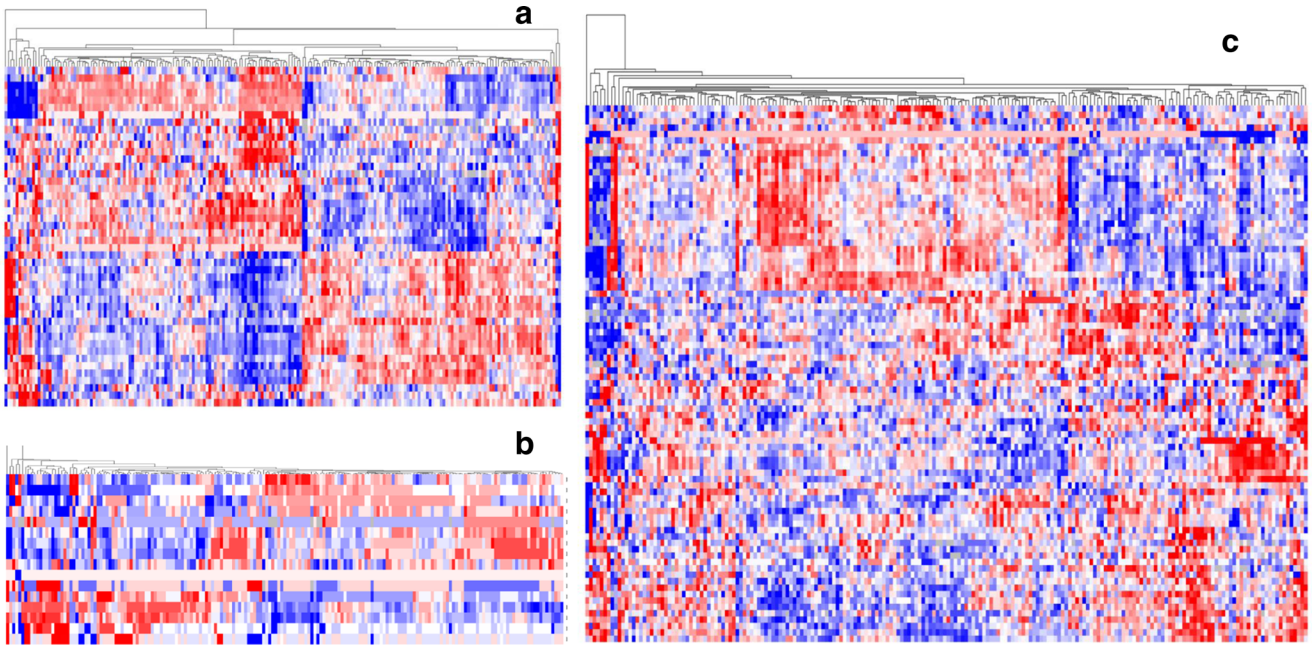

Fig. 2 Unsupervised hierarchical cluster analysis of the differentially expressed microRNAs. The microRNA expression levels in muscle-invasive bladder cancer tissues were compared between different groups according to **a** pathologic T (T3+T4 vs. T2), **b** pathologic N (N1–3 vs. N0), **c** histologic grade (high grade vs. low grade)

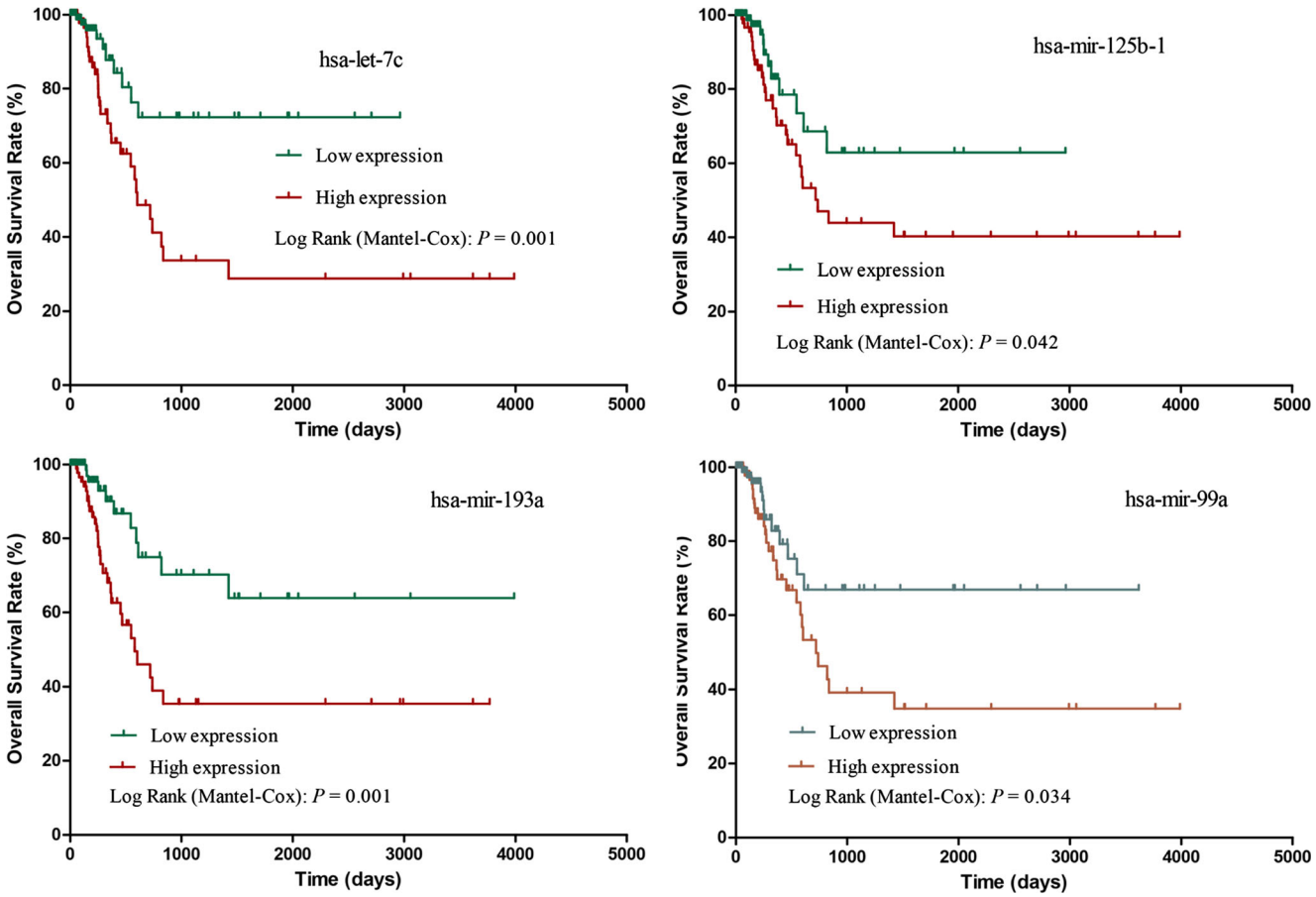

Fig. 3 Kaplan–Meier curve analysis of microRNAs for the survival in muscle-invasive bladder cancer patients. A total of four microRNAs were presented, including let-7c, mir-125b-1, mir-193a, and mir-99a

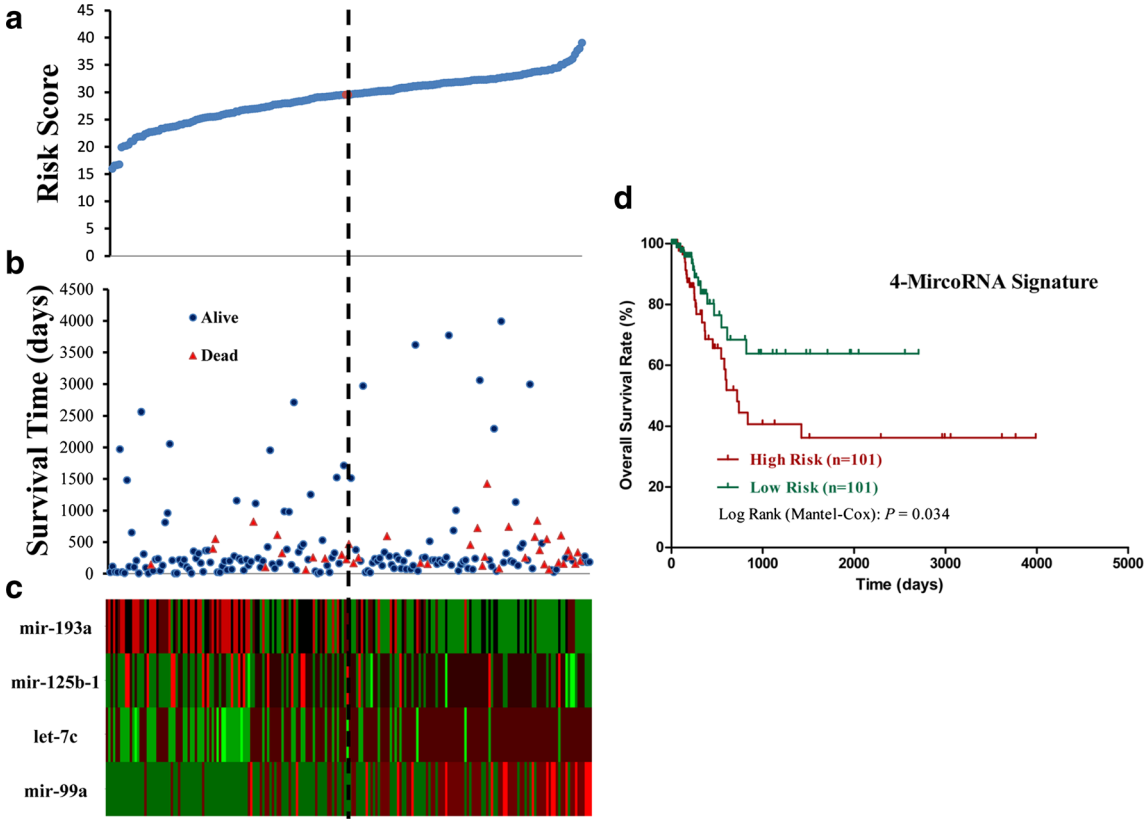

Fig. 4 The four-microRNA risk score analysis of muscle-invasive bladder cancer patients. **a** MicroRNA risk score distribution. **b** Patients' survival status and time. **c** Heat map of the miRNA expression profiles; *rows* represent miRNAs, and *columns* represent patients. The *black dotted line* represents the microRNA signature cutoff value (*median*) dividing patients into low-risk and high-risk groups. **d** Kaplan–Meier curve analysis of the microRNA signature for overall survival in 202 patients with muscle-invasive bladder cancer

carcinogenesis of metastatic medullary thyroid carcinoma [37] and to enhance both tumor growth and chemoresistance of liver cancer [38]. In BC, mir-193a has been proven to promote the multiple chemoresistance by targeting *HOXC9* and *LOXL4* genes [39, 40]. Overall, all these four miRNAs provided novel insights into the initiation and progression of MIBC, which could function as the potent prognostic parameters.

Some limitations of the current study should be acknowledged. First, as the metastasis status was unknown for 98 (48.5 %) patients and only 10 (5.0 %) low-grade MIBC subjects were included, these two previously established prognostic factors were excluded in the survival analysis, which could reduce the statistical robustness of the survival analysis. Second, partially due to the limited follow-up time with limited events, the four-miRNA signature did not pass the multivariate Cox regression analysis, and only pathologic T was proven as an independent prognostic factor. Third, the miRNA expression profiling was performed in MIBC tissues, which might limit the translational application of the generated

Table 2 Univariate analysis of parameters associated with overall survival in muscle-invasive bladder cancer patients

Variables	HR	95 % CI	*P* value
Age (≥68 vs. <68)	1.270	0.674–2.392	0.460
Gender (male vs. female)	0.905	0.451–1.816	0.779
Race (Caucasians vs. non-Caucasians)	0.723	0.221–2.364	0.592
Pathologic stage (III+IV vs. II)	2.815	1.181–6.710	**0.020**[a]
Pathologic T (T3+T4 vs. T2)	2.688	1.236–5.847	**0.013**[a]
Pathologic N (N1–3 vs. N0)	2.203	1.162–4.177	**0.016**[a]
Histologic subtype (papillary vs. non-papillary)	0.516	0.202–1.320	0.167
Risk score (high risk vs. low risk)	2.021	1.042–3.921	**0.037**[a]

HR hazard ratio, *95 % CI* 95 % confidential interval

[a] Statistical significant result (in bold)

four-miRNA signature in the clinical practice because of invasiveness and unavailability of specimens [41]. Fourth, this study was limited within the microarray analysis; as such, further clinical and biological studies should be conducted to validate our findings.

Conclusions

In summary, our study identified the specific miRNAs in relation with the progression and aggressiveness of MIBC and a miRNA signature consisting of let-7c, mir-125b-1, mir-193a, and mir-99a as a novel biomarker for MIBC prognostication.

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Conflicts of interest None

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